STUDIES ON THE OPTICAL RESOLUTION OF CARBOXYLIC ACID BY HYDROLASES IN ORGANIC SOLVENT SYSTEMS(Dissertation_全文)

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PREFACE

This is a thesis submitted by the author to Kyoto University for the degree of Doctor of Engineering. The studies collected here have been carried out under the direction of Professor Atsuo Tanaka in the Laboratory of Industrial Biochemistry, Department of Industrial Chemistry, Faculty of Engineering, Kyoto University during 1985-1992.

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INTRODUCTION

Enzymes as catalysts

Enzymes, which are proteins constructed from 20 kinds of amino acids, play important roles in metabolism of living systems, by catalyzing complicated chemical reactions. Up to now more than 2000 kinds of enzymes are known (1) and several hundreds of them are commercially available, including significant numbers in immobilized forms (2). In comparison with chemical catalysts, enzymes have the following features.

1) Enzymes are extremely versatile and catalyze broad spectra of reactions. There are enzyme-catalyzed equivalents for most types of organic reactions.

2) Enzyme-mediated reactions take place under mild conditions, such as ordinary temperature, atmospheric pressure, and pH value around neutrality. This minimizes problems of isomerization, racemization, epimerization, and rearrangement that often plague traditional methodology, and energy and resources can be saved.

3) Enzymes are highly efficient catalysts. The rates

of enzyme-promoted reactions can be greater than those of the corresponding uncatalyzed reactions by the factors of up to 10^{12} (3).

4) Enzymes are generally very selective in terms of the types of reactions catalyzed and with respect to the structure and stereochemistry of the substrates and products. These properties collectively constitute the specificity of an enzyme and are its most important feature for selective and asymmetric synthetic exploitation.

5) Enzymatic activity can be easily regulated by the reaction conditions: that is, catalytic activity may be strongly influenced by pH, temperature, pressure or concentrations of substrates, products or other species present in solution.

Because of their excellent catalytic features as described above, the application of enzymes to organic synthesis (4-7), analysis (8-13), and clinics (14-15) has been attracting remarkable attentions from all over the world.

However, some of the features of the enzymes may even be disadvantageous in practical use as catalysts. The high

selectivity of enzymes is liable to limit the application of enzymes to a narrow field. Enzymes are generally unstable, so believed to be deactivated by organic solvents.

Furthermore, the majority of enzymes require coenzymes such as NAD(P)H, ATP or coenzyme A. These cofactors are too expensive to be used in the stoichiometric amounts formally required. Accordingly, coenzyme-dependent enzymes can not be employed as catalysts without an efficient and inexpensive system for continuous in situ regeneration of the active forms of the cofactors.

In the meantime, hydrolases do not require coenzymes and, consequently, can be easily handled. Furthermore, hydrolases are readily available from commercial sources. Therefore, hydrolases are very attractive as target enzymes for the studies on the application of enzymes.

Enzymes in organic solvent systems

The ability of enzymes as mentioned above was noted in the field of synthetic organic chemistry. However, enzyme reactions, even in those cases which water-insoluble and lipophilic compounds are substrates, used to be carried out in aqueous reaction systems because biocatalysts were

believed to be unstable in organic solvents, unlike most ordinary chemical catalysts (16-17). With the recent development of enzyme technology, much wider application of biocatalysts is expected, including bioconversion of the compounds having a hydrophobic character. In such bioconversion systems it is often necessary to introduce organic solvents to enhance the solubility of reactants in water.

Introduction of appropriate organic solvents is very useful to construct homogeneous reaction systems containing lipophilic and water-insoluble substrates and to carry out such reactions continuously. Furthermore, organic solvents permit reactions otherwise impossible in water, such as synthetic reactions and group exchange reactions by hydrolases.

To introduce organic solvents in enzymatic reactions, one must have knowledge about the effects of organic solvents on properties and functions of enzymes (18-19).

Enzymes as well as other proteins maintain their structural conformation through intramolecular interactions among side chains of the component amino acids. Hydrophobic

side chains also contribute to such interactions. In general, enzyme molecules in aqueous solutions have both hydrophilic domains in contact with water and hydrophobic domains folded inside the molecules. When the polarity of the medium surrounding the enzyme molecules is reduced by adding organic solvents, the hydrophobic domains are liable to disperse, resulting in the unfolding of the molecules. Furthermore, hydrophobic interactions between the enzyme and substrate molecules are also disrupted. These facts indicate that suitably hydrated states of enzyme molecules should be maintained to keep the enzymes active and stable even when organic solvents are utilized in enzymatic reaction systems. It is also useful to suppress the unfolding of the molecules by immobilization, that is, by binding or interacting enzymes with appropriate supporting materials at multiple points (15-17, 20-21).

Organic solvents produce various physicochemical effects besides the above-mentioned effects on enzyme molecules, and the effects differ depending upon the kinds of organic solvents and the enzymes.

Zaks and Klibanov (22-24) have demonstrated that the use of dried lipases in organic solvents improved or changed

properties of enzymes such as thermostability and substrate specificity.

Thus organic solvents exhibit contradictory actions as stimulators and inhibitors for enzymatic reactions, depending on the kinds of enzymes and the types and concentrations of organic solvents.

It is, therefore, essential to select a suitable enzyme that exhibits an excellent catalytic activity in the organic solvent systems.

Application of hydrolases in organic solvent systems

Hydrolases are the easiest enzymes to handle because hydrolases do not require coenzymes and are readily available, as described before.

Furthermore, a broadly applicable class of enzymes to reactions in organic solvent systems is thought to be the hydrolases. They can be expected to retain a high degree of activity in organic solvent systems because they often operate at water-organic interfaces.

In organic solvent systems, hydrolases can also catalyze the condensation and group exchange reactions. One

Table 1. Several Examples of Hydrolases Applied in the Presence of Organic Solvents

Application	Solvent	Substrate	Product	Enzyme (source)	Ref.
Stereoselective hydrolysis	n-Heptane (water-saturated)	Menthyl succinate	Menthol	Rhodotorula minuta cells	25
Stereoselective esterification	Cyclohexane or iso-octane (water-saturated)	Menthol + acid	Menthyl ester	Lipase (Candida cylindracea)	26
	Benzene (water-saturated)	1-Tetradecanol + 2-(4-chloro-phenoxy)propanoic acid	1-Tetradecyl 2-(4-chloro-phenoxy)-propanoate	Lipase (Candida cylindracea)	27
	Benzene (water-saturated)	Hydroxyalkyltri-methylsilanes + 2-(4-chlorophenoxy)-propanoic acid	Trimethyl-silylalkyl 2-(4-chloro-phenoxy)-propanoates	Lipase (Candida cylindracea	28-29

Table 1. (continued)

Stereoselective trans-esterification	Methyl acetate	2-Substituted 1,3-propanediols + methyl acetate	2-Substituted 1,3-propane-diol mono-acetates	Carboxylesterase (porcine pancreas)	30
	2-Butanol (buffer-organic solvent two phase system) etc.	Tributyrin + 2-butanol etc.	Butyrate esters	Lipase (Candida cylindracea)	31
	1-Butanol	Methyl 2-(4-chloro-phenoxy)propano-ate + 1-butanol	Butyl 2-(4-chloro-phenoxy)-propanoate	Lipase (Candida cylindracea)	32
	Methyl propanoate (water-saturated)	3-Methoxy-1-butanol etc. + methyl propanoate	Propanoates	Carboxylesterase (hog pancreas)	31
Stereoselective lactonization	Diethyl ether	γ-Substituted γ-hydroxybutyrates	γ-Substituted γ-butyrolactones	Lipase (porcine pancreas)	33

Table 1. (continued)

Stereoselective poly-condensation	Toluene	Bis(2-chloroethyl) adipate + 2,4-pentanediol	Ester oligomers	Lipase (porcine pancreas)	34
Stereoselective amide synthesis	3-Methyl-3-pentanol *etc.*	Amines + trifluoro-ethyl butyrate	Amides	Subtilisin (*Bacillus licheniformis*)	35
Stereoselective thiotrans-esterification	*n*-Hexane	Methyl 3-(acetyl-thio)-2-methyl-propanoate + 1-propanol	Methyl mercapto-2-methyl-propanoate	Lipase (porcine pancreas)	36
Regioselective acylation	Tetrahydro-furane (containing 20 % acetone)	6-*O*-Butyryl sugars + trichloroethyl butyrate	3,6-Di-*O*-butyryl sugars	Lipases (*Chromobacterium viscosum*, *Aspergillus niger etc.*)	37
	Acetonitrile	L-Phenylalanyl-L-lysine *tert*-butyl ester + trifluoro-ethyl acetate	L-Phenylalanyl-*N*-ε-acetyl-L-lysine *tert*-butyl ester	Lipase (*Pseudomonas* sp.)	38

Table 1. (continued)

Regioselective trans-esterification	Petroleum ether	Palm oil + saturated fatty acids	Reformed palm oil	Lipases (*Aspergillus niger*, *Rhizopus niveus*)	39
	n-Hexane (water-saturated)	Olive oil + saturated fatty acids	Reformed olive oil	Lipases (*Geotrichum candidum*, *Rizopus delemar*)	39-41
Peptide synthesis	Ethyl acetate	Carbobenzyloxy-phenylglycine + alanine propyl ester *etc*.	Various peptides	Lipase (*Candida cylindracea*)	42

of the major application of hydrolases in organic solvent systems is, therefore, the regio- or stereoselective condensation or group exchange reactions (Table 1) (25-42).

However, the number of hydrolases applied has so far been limited and it has been required further to screen the hydrolases having a high activity in organic solvent systems. Furthermore, there have been few systematic studies concerning the stereoselective esterification of racemic carboxylic acids, although the optically pure carboxylic acids, especially halogen-containing carboxylic acids, should be useful as chiral building blocks and bioactive compounds such as herbicide (31).

Continuous operation of bioconversion in organic solvent systems

Biochemical processes using organic solvents are useful processes. From the industrial view point, construction of continuously operated biochemical systems for the efficient production of useful substances are, however, very important. Continuously operated systems have the following features compared with batchwise systems (43-44).

1) It is possible to save time.

2) Automatic control is easy.

3) Reaction conditions are easily kept stable.

4) Quality of products produced is easily maintained.

Therefore, many examples of continuously operated bioreactors using immobilized enzymes and immobilized microbial cells in aqueous systems have been reported (45-48). There have, however, so far been only a few reports of continuously operated bioreactors using organic solvents (39, 49-53) and had been no study on a continuously operated systems applying stereoselectivity of biocatalysts, in spite of importance of bioconversion of various lipophilic compounds in organic solvent systems.

The sure retention of enzymes in bioreactor and the improvement of stability of enzymes are very important for the efficient long-term continuous operation of bioreactors. Therefore, immobilization has so far been essential technique. However, immobilization sometimes causes the deactivation of enzymes and the limitation of diffusion of substrates.

In general, enzyme proteins are insoluble in organic solvents but can act in a suspended free state (23-24, 54).

This fact suggests that it is possible to retain enzymes in reactors without immobilization. To overcome the disadvantages of immobilization as mentioned above, it is necessary to construct an efficient new bioreactor by applying the such insolubility of enzymes in organic solvents.

Introduction of organosilicon compounds into bioconversion systems

Increasing interest has been focused on the application of enzymes in organic solvent systems. There are actually many examples as shown in Table 1. However, many problems remain to be solved in the enzymatic processes. For example, it is often quite difficult to construct a highly stereoselective enzymatic reaction system with a large reaction rate, a very important prerequisite for industrial application of enzymes, by the use of conventional compounds as substrates.

To break through these problems, to raise the potential of enzymes, and to extend the application of enzymes, the use of unconventional compounds as substrates would be

effective. Especially, the use of organosilicon compounds as substrates is very attractive.

Silicon which is the second most abundant element in the Earth's crust (55-57) belongs to the same group as carbon which is one of the most fundamental elements of organisms, but silicon is different from carbon in characters (55). Silicon always appears as being markedly more electropositive and has longer covalent radius than carbon. Whereas silicon's bonds to oxygen and fluorine are stronger than the corresponding bonds between carbon and these elements, its bonds to carbon and hydrogen are week. Furthermore, silicon has the valence p- and empty d-orbitals. These differences between silicon and carbon are expected to cause the difference between organosilicon compounds and the corresponding carbon compounds in the enzymatic recognition, and may break through the problems in the case of conventional carbon compounds.

Silicon also plays important roles in the biosphere and is present in mainly living organisms especially at lower stage of evolutionary development: silicate bacteria, the simplest algae, and spore plants, in fact contain very large amounts of silicon (56-58). Recent researches indicate that

silicon is also important for the higher plants as well as in the organisms of the higher animals and man (59-66), although its actual biochemical mode of action is at present not unambiguously defined. It seems, however, that natural biochemical processes mainly involve silicon bonded to oxygen whereas organosilicon compounds containing Si-C bonds have not been detected (55-57). Therefore, the effects of organosilicon compounds on organisms and/or enzymatic recognition are very interesting.

However, only a few results have been reported on the bioconversion of organosilicon compounds (28-29, 67-69). Hitherto, there has been no study on the ability of organosilicon compounds to break the limit of conventional substrates and on the modes of enzymatic recognition for organosilicon compounds as compared with conventional substrates.

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SYNOPSIS

CHAPTER 1. Screening of Hydrolases Having High Esterification Activity in Organic Solvent and Effects of Reaction Conditions on Enzyme Activity

Considerable interest has been focused on the potential of enzymes in synthetic organic chemistry. It is essential to introduce organic solvents into the reaction systems to use enzymes in organic synthesis because many organic compounds are lipophilic or water-insoluble. However, enzymes are often denatured in the presence of organic solvents, resulting in the loss of catalytic activity. It is, therefore, important to select a suitable enzyme that exhibits an excellent catalytic activity in organic solvent systems.

In CHAPTER 1, 50 different hydrolases are studied for the screening of enzymes having high esterification activity towards citronellol (3,7-dimethyl-6-octen-1-ol) and 5-phenylpentanoic acid in an organic solvent. Among these

hydrolases, as many as 22 hydrolases exhibited the high esterification activity in the organic solvent. Lipase OF 360 from Candida cylindracea was selected for further studies on the effects of reaction conditions on the enzyme activity because of its availability and activity retention even after immobilization. Hydrophobic water saturated-isooctane was found to be the most suitable organic solvent, whereas polar organic solvents such as acetone and chloroform caused reversible inactivation of the enzyme. Entrapment with hydrophobic polyurethane gel, PU-3, significantly enhanced the operational stability of the lipase in the organic solvent.

CHAPTER 2. Stereoselective Esterification of Halogen-Containing Carboxylic Acids by Lipases in Organic Solvents

High stereoselectivity is the most attractive characteristic of enzymes. Then, one of the major application of enzymes in organic solvent systems is the optical resolution of racemic compounds. Hitherto, most

studies have mainly been performed on the optical resolution of racemic alcohols and their esters, but systematic studies concerning the stereoselective esterification of racemic acids have been very limited.

CHAPTER 2 deals with the optical resolution of carboxylic acids containing a halogen atom with stereoselective esterification by Celite-adsorbed hydrolases in organic solvents at 30°C with shaking. Lipase OF 360 from Candida cylindracea was found to stereoselectively esterify 2-(4-chlorophenoxy)propanoic acid, the (R)-enantiomer (d-isomer) of which is an important herbicide, in water-saturated benzene. Furthermore, the effects of alcohol chain length on stereoselectivity as well as reaction rate of esterification of 2-(4-chlorophenoxy)propanoic acid with lipase OF 360 were studied. The results showed that the alcohol chain length remarkably affected the stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid. It was found that longer-chain alcohols took much longer to reach 50 % conversion than shorter-chain alcohols. On the other hand, the longer was the alcohol chain length, the higher the stereoselectivity observed. 1-Tetradecanol served as a suitable substrate for optical resolution of 2-(4-

chlorophenoxy)propanoic acid, although the reaction rate was moderate.

CHAPTER 3. Long-Term Continuous Production of Optically Active 2-(4-Chlorophenoxy)propanoic Acid by Celite-Adsorbed Yeast Lipase in Organic Solvent Systems

Continuously operated biochemical systems for the production of useful compounds are considerably important from an industrial viewpoint. In application of enzymes in aqueous systems, many examples of continuously operated bioreactors have been reported.

For the bioconversion of various lipophilic or water-insoluble compounds, it is essential to introduce organic solvents into reaction systems as mentioned before. However, there have so far been only a few reports on continuous bioconversion in organic solvent systems and no study on continuously operated systems applying stereoselectivity of enzymes in organic solvent systems in spite of many works on bioconversion in organic solvent systems.

CHAPTER 3 deals with long-term continuous production of optically active 2-(4-chlorophenoxy)propanoic acid by stereoselective esterification with 1-tetradecanol by Celite- adsorbed lipase OF 360 in organic solvent systems. The productivity was affected by the water content of the Celite-adsorbed lipase. There was the optimal water content for preparation of the adsorbed lipase. Water-saturated carbon tetrachloride-isooctane (2,2,4-trimethylpentane) (8:2, v/v) was a suitable organic solvent for continuous operation. The particle size of Celite did not affect the productivity. Under the optimized conditions (hydrolase, 10 g lipase OF 360 (Celite-adsorbed); organic solvent, water-saturated carbon tetrachloride-isooctane (8:2, v/v); concentration of substrates, 100 mM; flow rate, 86.4 $ml.day^{-1}$; reaction temperature, $30^{o}C$), highly optically active (S)-enantiomer of 2-(4-chlorophenoxy)propanoic acid was continuously obtained by packed-bed column reactor for 34 days. The treatment of the reactor with acetone to remove water formed made it possible to restore the productivity and extend the period of continuous operation for further 29 days.

CHAPTER 4. Construction of Non-Support Bioreactor for Optical Resolution of 2-(4-Chlorophenoxy)-propanoic Acid in Organic Solvent Systems

From an industrial viewpoint, construction of efficient bioreactors for continuous production of useful compounds is one of the most important subjects.

CHAPTER 4 deals with the construction of a new type of a column reactor packed with free non-supported lipase OF 360, "non-support bioreactor", by applying the insolubility of enzymes in organic solvents to achieve the efficient continuous optical resolution of 2-(4-chlorophenoxy)propanoic acid by the enzymatic stereoselective esterification with 1-tetradecanol. Non-support bioreactor exhibited four-fold higher productivity than that of a column reactor packed with the Celite-adsorbed lipase (adsorbed bioreactor). However, the optical purity of the remaining (S)-acid was low even when the conversion was kept at approximately 50 %. Lipase recovered from the non-support bioreactor after continuous operation retained the original stereoselectivity in a

batchwise reaction. Therefore, semi-continuous operation was conducted by recycling the substrate solution at a high flow rate. The semi-continuously operated non-support bioreactor showed high stereoselectivity and ten times the productivity compared with the adsorbed bioreactor. The non-support bioreactor had many advantages, for example, simplicity of preparation, small volume of reactor, and low cost. Furthermore, it provided high activity without the inactivation of enzymes accompanying immobilization and without interference to diffusion caused by the supports, resulting in a high productivity based on the reactor volume.

CHAPTER 5. Efficient Optical Resolution of 2-(4-Chlorophenoxy)propanoic Acid by the Use of Organosilicon Compounds as Substrates of Lipase.

There are many reports of enzymatic stereoselective reactions in organic solvent systems. The author has also successfully curried out the optical resolution of 2-(4-chlorophenoxy)propanoic acid by enzymatic stereoselective

esterification with 1-tetradecanol in an organic solvent and revealed that the chain-length of alcohols as the acyl acceptors markedly affected both the reaction rate and the stereoselectivity. However, it has been difficult to construct a highly stereoselective bioconversion system with a high reaction rate by the use of conventional compounds as substrates. Then, the use of unconventional compounds, such as organosilicon compounds, as substrates would be very attractive in order to break through the above problem and raise the potential of enzymes. Organosilicon compounds are very attractive as target because organosilicon compounds play important roles in organic chemistry. Fundamental studies on the recognition and reaction of enzymes toward such unconventional compounds are also very interesting and important.

In this chapter, the author attempted to use organosilicon compounds ($Me_3Si(CH_2)_nOH$) as substrates to achieve efficient optical resolution of 2-(4-chlorophenoxy)propanoic acid and discussed about the enzymatic recognition of organosilicon compounds comparing with the corresponding carbon compounds. Organosilicon

compounds served as good substrates for lipase OF 360 in the stereoselective esterification of the (R)-enantiomer of 2-(4-chlorophenoxy)propanoic acid in water-saturated benzene. In particular, trimethylsilylmethanol (n = 1) enabled rapid and highly stereoselective esterification which was difficult with conventional substrates such as its corresponding carbon compound. On the other hand, there was no difference between 2-trimethylsilylethanol (n = 2) and its carbon analogue in the reaction rate and the stereoselectivity. These phenomena observed could be explained based on the properties of silicon atom such as low electronegativity and big atomic radius compared with carbon atom.

CHAPTER 1. Screening of Hydrolases Having High Esterification Activity in Organic Solvent and Effects of Reaction Conditions on Enzymatic Activity

INTRODUCTION

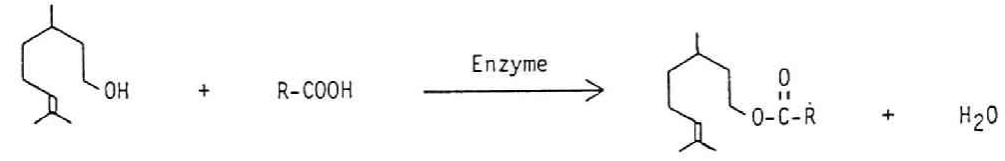
Recently, in both the academic and the industrial worlds, a considerable interest has been focused on the application of the great potential of enzymes to synthetic organic chemistry.

To apply enzymes to organic synthesis, it is essential to introduce organic solvents into the reaction systems in order to improve the poor solubility of lipophilic or water-insoluble organic compounds in water. Furthermore, organic solvents are effective to make it possible to conduct new reactions impossible in water, such as synthetic reactions and group exchange reactions by hydrolytic enzymes. Zaks and Klibanov (1-3) have demonstrated that the use of dried lipases in organic solvents improved their properties such as thermostability and substrate specificity. However,

enzymes are often liable to be denatured in the presence of organic solvents, resulting in the loss of their catalytic activity (4). Therefore, it will be essential to select an adequate enzyme which exhibits an excellent catalytic activity in organic solvents. Furthermore, to render enzymes so selected resistant to organic solvents, immobilization seems to be the most general and promising technique (5-7).

Bioconversion of various lipophilic compounds in organic solvent systems by using immobilized biocatalysts including lipases have been successfully carried out (8-9). Nevertheless, the number of enzymes applied to bioconversion of lipophilic compounds in organic solvents has so far been limited.

In this chapter, fifty different hydrolases (Table 1) were examined for the activity concerning esterification of citronellol (Fig. 1) in organic solvent and the effects of several factors on the enzyme activity in organic solvent were studied with the selected enzyme, Candida cylindracea lipase.



Citronellol

Fig. 1. Esterification of citronellol.

MATERIALS AND METHODS

Enzymes

Enzymes used in this study were as follows: 37 kinds of lipases from yeast, fungi, bacteria, porcine pancreas, and wheat germ; 5 kinds of lipoprotein lipases from bacteria; 3 kinds of esterases from porcine liver; and 5 kinds of cholesterol esterases from bacteria etc. Some of the enzymes were obtained from commercial sources and the others were kindly donated by several companies (Table 1).

Table 1. Hydrolases tested

Hydrolase	Source	Maker
Lipase MY	*Candida cylindracea*	Meito Sangyo
Lipase OF 360	*Candida cylindracea*	Meito Sangyo
Lipase Type VII	*Candida cylindracea*	Sigma
Lipase AY	*Candida cylindracea*	Amano Seiyaku
Lipase 643 335	*Candida cylindracea*	Boehringer
Lipase 205-33	*Candida cylindracea*	Nacalai Tesque
Candida lipase	*Candida cylindracea*	Cooper Biomedical
Lipase	*Candida paralipolytica*	Meito Sangyo
Lipase CE	*Humicola langinosa*	Amano Seiyaku
Lipase M-AP 10	*Mucor*	Amano Saiyaku
Lipase	*Mucor lipolyticus*	Meiji Seika
Lipase SP225	*Mucor miehei*	Novo-Nordisk
Lipase (Talipase)	*Rhizopus delemar*	Tanabe Seiyaku
Lipase Fine grade	*Rhizopus delemar*	Seikagaku Kogyo
Lipase F-AP	*Rhizopus javanicus*	Amano Seiyaku
Lipase (Olipase 2S)	*Rhizopus japonicus*	Osaka Saiken

Table 1. (continued)

Lipase (Olipase 4S)	*Rhizopus japonicus*	Osaka Saiken
Lipase Saiken 100	*Rhizopus japonicus*	Osaka Saiken
Lipase Saiken 50	*Rhizopus japonicus*	Osaka Saiken
Lipase	*Aspergillus niger*	Nagase Sangyo
Lipase AP 6	*Aspergillus* sp.	Amano Seiyaku
Lipase G	*Penicillium* sp.	Amano Seiyaku
Lipase P-1	*Phycomyces nitens*	Takeda Yakuhin
Lipase PN	*Phycomyces nitens*	Wako
Lipase Pure grade	*Geotrichum candidium*	Seikagaku Kogyo
Lipase	*Achromobacter* sp.	Meito Sangyo
Lipase	*Alkaligenes* sp.	Meito Sangyo
Lipase	*Arthrobacter ureafaciens*	Godo Shusei
Lipase T-01	*Chromobacterium viscosum*	Toyo Jozo
Lipase Blend	Fungus & Bacterium	Scripps Lab.
Lipase (Steapsin)	Hog pancreas	Tokyo Kasei
Lipase Type II	Hog pancreas	Sigma
Lipase Type II	Porcine pancreas	Sigma
Lipase 644072	Porcine pancreas	Boehringer

Table 1. (continued)

Lipase II	Porcine pancreas	Cooper Biomedical
Lipase	Pancreas	Wako
Lipase Type I	Wheat germ	Sigma
Lipoprotein lipase Type A	Pseudomonas sp.	Toyobo
Lipoprotein lipase LPL	Pseudomonas sp.	Amano Seiyaku
Lipoprotein lipase	Pseudomonas sp.	Sigma
Lipoprotein lipase	Pseudomonas sp.	ICN Nutritional
Lipoprotein lipase LPL-Oriental	Bacterium	Oriental Yeast
Esterase Type I	Porcine liver	Sigma
Esterase Type II	Porcine liver	Sigma
Esterase 104 698	Hog liver	Boehringer
Cholesterol esterase Type A	Pseudomonas sp.	Toyobo

Table 1. (continued)

Cholesterol esterase CHE Amano II	Pseudomonas sp.	Amano Seiyaku
Cholesterol esterase III	Schizophyllum commune	Toyobo
Cholesterol esterase CE	Pancreas	Oriental Yeast
Cholesterol esterase T-18	Microorganism	Toyo Jozo

Synthetic resin prepolymers

A hydrophilic photo-crosslinkable resin prepolymer, ENT-4000 (10) (the number indicates the approximate molecular weight of poly(ethylene glycol) skeleton), and a hydrophobic photo-crosslinkable resin prepolymer, ENTP-4000 (11) (the number indicates the approximate molecular weight of poly(propylene glycol) skeleton), were prepared by Kansai Paint Co., Ltd., Tokyo, Japan. Two kinds of water-miscible urethane prepolymers, PU-3 and PU-6 (12), were provided by Toyo Tire and Rubber Industry Co., Osaka, Japan. PU-3 gives hydrophobic gels, while PU-6 gives hydrophilic gels.

Chemicals

Citronellol was obtained from Nacalai Tesque, Ltd., Kyoto, Japan. 5-Phenylpentanoic acid was purchased from Aldrich Chemical Co., Milwaukee, WI, USA. Celite (No. 535) was obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Other chemicals were also obtained from commercial sources.

Adsorption of hydrolases on Celite

For the esterification of citronellol in organic solvents, 100 mg of enzyme dissolved in 100 µl of deionized water was mixed thoroughly with 250 mg of Celite. In the case of screening test, 30 mg of enzyme was applied after adsorption on Celite. Celite-adsorbed lipase thus obtained was designated as C-lipase.

Immobilization of lipases with hydrophilic photo-crosslinkable resin prepolymer

ENT-4000 (0.5 g) was mixed with 5 mg of a photo-sensitizer, benzoin ethyl ether, and 50 µl of deionized water. The mixture was melted by warming at 60°C, cooled to room temperature and then 100 mg of free lipase in 50 µl of deionized water was added to the molten mixture. The mixture was gelled by a short illumination with near-UV light and gel formed (thickness, ca. 0.5 mm) was cut into small pieces (5 x 5 mm) (9, 13). In some cases, C-lipase corresponding to 100 mg of lipase was entrapped by the same procedure.

Immobilization of lipases with hydrophobic photo-crosslinkable resin prepolymer

ENTP-4000 (0.5 g) and 5 mg of benzoin ethyl ether were dissolved in 400 µl of water-saturated cyclohexane. C-lipase (100 mg of lipase) was added to the mixture and entrapment was carried out as described above.

Immobilization of lipases with urethane prepolymers

The typical method was as follows: PU-3 or PU-6 (0.5 g) was melted at 60°C and cooled to room temperature. The molten prepolymer was mixed quickly with free lipase (100 mg) dissolved in 300 µl of deionized water or C-lipase (100 mg of lipase) (PU-3-C-lipase or PU-6-C-lipase) (12, 14).

Enzyme reactions

Unless stated otherwise, the reactions were carried out at 30°C with shaking (120 strokes.min^{-1}). The reaction mixture was composed of C-lipase or immobilized lipase (corresponding to 100 mg of lipase) and 10 ml of water-saturated cyclohexane or isooctane containing 130 mM citronellol and 100 mM 5-phenylpentanoic acid.

Analysis

Alcohol and ester were determined gas chromatographically by using a 1.0 m glass column packed with silicon AN 600 supported on chromosorb W AW-DMCS (carrier gas, He; flow rate, 60 $ml.min^{-1}$; injection temperature, 300°C). When the temperature of column was increased from 60 to 265°C at $20°C.min^{-1}$, the retention times observed were 2.1 min for citronellol and 7.5 min for citronellyl 5-phenylpentanoate.

RESULTS AND DISCUSSION

Screening of active hydrolases

Fifty hydrolases were tested (Table 1) for the citronellol esterification activity in water-saturated cyclohexane. It was found that 22 hydrolases had a high activity under the conditions employed (Table 2). Among the active hydrolases, lipase OF 360 from Candida cylindracea, lipase Saiken 100 from Rhizopus japonicus, and lipase (Steapsin) from hog pancreas were selected as the hydrolases to be used for further studies because these hydrolases are readily

Table 2. Hydrolases having a high activity of esterification of citronellol in organic solvent

Hydrolase	Source	Maker	Conversion
Lipase OF 360	Candida cylindracea	Meito Sangyo	100
Lipase 643 335	Candida cylindracea	Boehringer	100
Lipase CE	Humicola langinosa	Amano Seiyaku	100
Lipase SP225	Mucor miehei	Novo-Nordisk	99
Lipase Saiken 100	Rhizopus japonicus	Osaka Saiken	100
Lipase P-1	Phycomyces nitens	Takeda Yakuhin	91
Lipase T-01	Chromobacterium viscosum	Toyo Jozo	100
Lipase Blend	Fungus & Bacterium	Scripps Lab.	99

Table 2. (continued)

Lipase (Steapsin)	Hog pancreas	Tokyo Kasei	99
Lipase Type II	Hog pancreas	Sigma	99
Lipase Type II	Porcine pancreas	Sigma	97
Lipase 644072	Porcine pancreas	Boehringer	92
Lipase II	Porcine pancreas	Cooper Biomedical	100
Lipase	Pancreas	Wako	91
Lipoprotein lipase Type A	*Pseudomonas* sp.	Toyobo	98
Lipoprotein lipase LPL	*Pseudomonas* sp.	Amano Seiyaku	100
Lipoprotein lipase	*Pseudomonas* sp.	Sigma	100
Lipoprotein lipase	*Pseudomonas* sp.	ICN Nutritional	100

Table 2. (continued)

Cholesterol esterase Type A	*Pseudomonas* sp.	Toyobo	100
Cholesterol esterase CHE Amano II	*Pseudomonas* sp.	Amano Seiyaku	100
Cholesterol esterase CE	Pancreas	Oriental Yeast	100
Cholesterol esterase T-18	Microorganism	Toyo Jozo	100

The reactions were carried out for 48 h in water-saturated cyclohexane (3.0 ml) with Celite-adsorbed hydrolases (30 mg of hydrolase). Other conditions were the same as those described in the text.

available.

Effect of supports

Lipase OF 360 entrapped with hydrophobic synthetic resin prepolymers, PU-3 and ENTP-4000, exhibited relatively high activity, while lipase Saiken 100 and lipase (Steapsin) were deactivated by entrapment with synthetic resin prepolymers (Table 3). Especially, lipase OF 360 entrapped with PU-3 after Celite adsorption had a high activity.

In the case of steroid dehydrogenation, a close relationship was observed between the activity of entrapped biocatalysts and hydrophobic properties of substrates and gels entrapping biocatalysts. This phenomenon was reasonably explained by the partition of substrates between the gels and reaction solvent (15). Such effect of gel hydrophobicity was also observed in the hydrolysis of menthyl succinate by yeast cells entrapped in polyurethane gels (16). In the case of the esterification of citronellol with 5-phenylpentanoic acid by entrapped lipase OF 360, similar results were also obtained.

Table 3. Effect of supports for lipases on ester formation from citronellol

Support	Lipase Saiken 100	Lipase (Steapsin)	Lipase OF 360
	(μmol.h^{-1}.mg lipase^{-1})		
Celite	0.44	0.58	0.40 - 1.78
PU-3	0.03	0.15	0.63
Celite-PU-3	0.01	0.16	0.86 - 1.11
PU-6	0.02	0.07	0.02
Celite-PU-6	----[a]	----[a]	0.05
Celite-ENTP-4000	0.22	0.37	0.66
ENT-4000	----[a]	----[a]	nil
Celite-ENT-4000	----[a]	----[a]	0.01

The reactions were carried out in water-saturated cyclohexane (10 ml) with 100 mg of lipases adsorbed on Celite or entrapped with each prepolymer with or without Celite adsorption in the presence of 100 µl of deionized water. Other conditions were the same as those described in the text.

[a] Not tested.

Effect of water content in support

Water is not only another product of esterification reactions, but water in supports used for immobilization of enzymes also hinders the diffusion of hydrophobic substrates. Therefore, it is essential to remove water from the reaction systems to carry out esterification efficiently. However, the author has found a certain amount of water in supports (about 0.35 µl.mg immobilized enzyme preparation^{-1}) is essential for the maximum enzyme activity (Fig. 2). From these results, 300 µl (corresponding to 0.35 µl.mg immobilized enzyme preparation^{-1}) of water was used to prepare the entrapped enzyme from 0.5 g of PU-3, 250 mg of Celite, and 100 mg of lipase OF 360 (PU-3-C-lipase).

Effect of organic solvents

To construct homogeneous reaction systems containing citronellol and 5-phenylpentanoic acid and to make lipases serve for the synthesis of the ester, it is essential to use organic solvents. In the esterification of citronellol by lipase OF 360, non-polar solvents, especially isooctane, gave an excellent result, while no activity was observed in

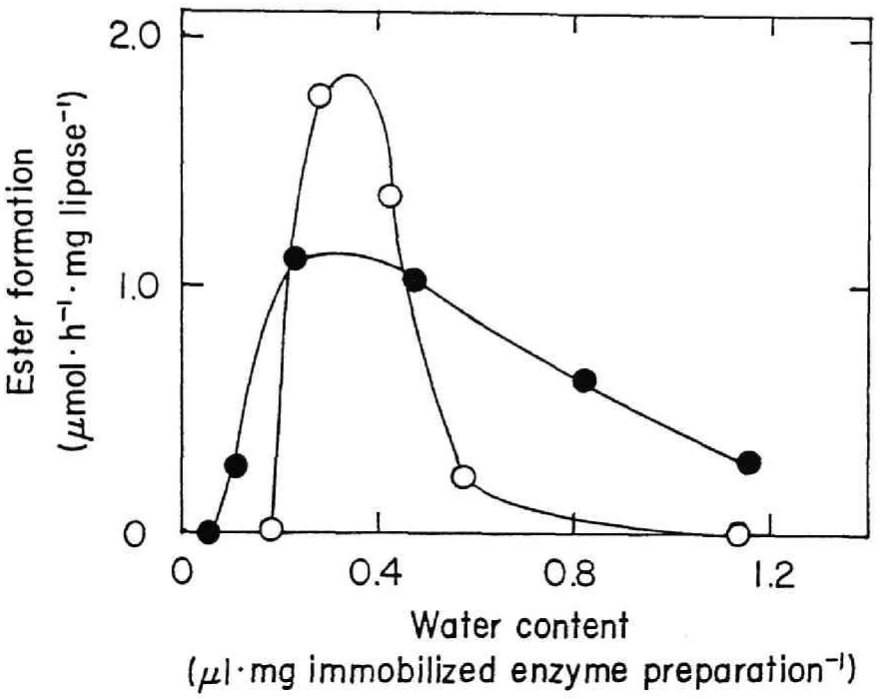


Fig. 2. Effect of water content on esterification of citronellol.

Water content was controlled by the amount of deionized water used to dissolve 100 mg of lipase OF 360. The reactions were carried out in water-saturated cyclohexane (10 ml) with C-lipase (350 mg) (○) prepared from 100 mg of lipase and 250 mg of Celite or with PU-3-C-lipase (850 mg) (●) prepared from 100 mg of lipase, 250 mg of Celite, and 0.5 g of PU-3, as described in the text.

Table 4. Effect of solvents on esterification of citronellol and partition coefficients of the substrates

Solvent	Ester formation ($\mu mol.h^{-1}.mg\ lipase^{-1}$)		Partition coefficient	
	C-lipase	PU-3-C-lipase	Citronellol	5-PPA
Acetone	0	0	1.5	1.4
Methanol	0	0	1.5	1.7
Chloroform	0	0	1.2	2.0
Tetrahydrofuran	0	0	---[a]	---[a]
Dioxane	0	0	---[a]	---[a]
Methyl isobutyl ketone	0	0	---[a]	---[a]
Carbon tetrachloride	0.31	0.24	1.7	2.9
Benzene	0.05	0.02	1.3	2.2
n-Hexane	1.37	0.38	2.9	10.4
Cyclohexane	1.33	0.85	2.9	7.0
Isooctane	2.18	0.95	3.0	12.9

(Footnote of Table 4)

Partition coefficients (ratio of concentration in gels to that in external solvent) of citronellol and 5-phenylpentanoic acid (5-PPA) were estimated with PU-3 gels (prepared from 250 mg of Celite, 300 µl of deionized water, and 0.5 g of PU-3) (22, 33). The enzymatic reactions were carried out with C-lipase OF 360 or PU-3-C-lipase OF 360 as described in the text. Water-immiscible solvents were used after saturated with water and water-miscible solvents were employed without addition of water.

[a] Not tested.

polar solvents, such as acetone, methanol, chloroform, tetrahydrofuran, 1,4-dioxane, and methyl isobutyl ketone (Table 4), as the cases of the reactions reported previously (8, 15-17).

As mentioned above, activity of gel-entrapped biocatalysts was, in general, correlated closely to the partition of substrates between the gels and reaction solvents. However, partition coefficients (ratio of concentration of a compound in gels to that in external solvent) of both substrates did not necessarily reflect the activity of PU-3-C-lipase when different solvents were employed as reaction solvents (Table 4). Especially, the ester formation was not observed in polar solvents in spite of relatively high partition coefficients of substrates. These results indicated strongly that polar solvents themselves gave an unfavourable effect to the PU-3-C-lipase. Recently, Takahashi et al. (18) and Kazandjian et al. (19) have reported that enzymes treated with hydrophilic solvents could not show the catalytic activity even in hydrophobic solvents when no extra water was added. Based on these observations, they supposed that water molecules critical

for the enzymatic activity were stripped by hydrophilic solvents, resulting in enzyme inactivation. However, the author has found that PU-3-C-lipase treated with acetone is very active on the esterification of citronellol in isooctane whether extra water was added or not (Table 5). Similar results were also obtained when chloroform was used as a solvent instead of acetone, but methanol inactivated PU-3-C-lipase irreversibly. The affinity of methanol toward lipase is presumably so strong that lipase can not accept a substrate having a hydroxyl group, since methanol is an alcohol. These results indicate that the water essential to the enzymatic activity appears not to be stripped completely by hydrophilic solvents and that inactivation of enzymes by hydrophilic solvents is often reversible.

Effect of acyl donors

For the ester formation catalyzed by lipases, chain-length of acids used as acyl donors seriously affects the yield of esters (8, 20-21). As shown in Table 6, fatty acids having longer chain were in general excellent acyl donors for the esterification of citronellol, while shorter chain

Table 5. Effect of treatment with hydrophilic solvents on the activity of entrapped lipase OF 360

Treatment (24 h) with	Addition of water (300 µl)	Enzyme reaction in	Ester formation ($\mu mol.h^{-1}.mg\ lipase^{-1}$)
Acetone	+	Isooctane	2.02
Acetone	-	Isooctane	2.02
Methanol	+	Isooctane	0
Methanol	-	Isooctane	0
Chloroform	+	Isooctane	1.06
Chloroform	-	Isooctane	1.66
Water-saturated isooctane	-	Water-saturated isooctane	2.19
Water-saturated isooctane	-	Isooctane	2.41

The enzymatic reactions were carried out in isooctane or water-saturated isooctane with PU-3-C-lipase OF 360 as described in the text after the gels were treated in each organic solvent for 24 h at 30°C and washed with isooctane or water-saturated isooctane. In some cases, 300 µl of deionized water was added to the solvent-treated enzyme, before the enzymatic reactions.

fatty acids were not good substrates. 2,2-Dimethylpropanoic acid and 3-phenylpropanoic acid did not serve as substrates probably due to the steric hindrance. A low activity on octadecanoic acid might be resulted from a poor solubility of octadecanoic acid in water-saturated isooctane.

Repeated use of lipases

C-lipase and PU-3-C-lipase were used repeatedly in batchwise reactions at 20, 30 or 40°C. Although C-lipase showed a high activity in first batch at 40°C, the activity decreased rapidly probably due to inactivation or leakage of enzymes (Fig. 3). On the other hand, PU-3-C-lipase retained a high activity for a long period. Especially, stability of PU-3-C-lipase at 30°C was excellent.

Table 6. Effect of acyl donors on esterification of citronellol

Acyl donor	Ester formation ($\mu mol.h^{-1}.mg\ lipase^{-1}$)	
	C-lipase	PU-3-C-lipase
Acetic acid	0.24	0.05
2,2-Dimethylpropanoic acid	0	0
Pentanoic acid	1.05	0.90
Heptanoic acid	2.18	1.60
3-Phenylpropanoic acid	0.46	0.18
5-Phenylpentanoic acid	2.77	0.73
Tetradecanoic acid	2.27	1.46
Octadecanoic acid	1.48	0.85
cis-9-Octadecenoic acid	8.53	5.00

The reactions were carried out in water-saturated isooctane including 100 mM of each acyl donor with C-lipase OF 360 or PU-3-C-lipase OF 360 as described in the text.

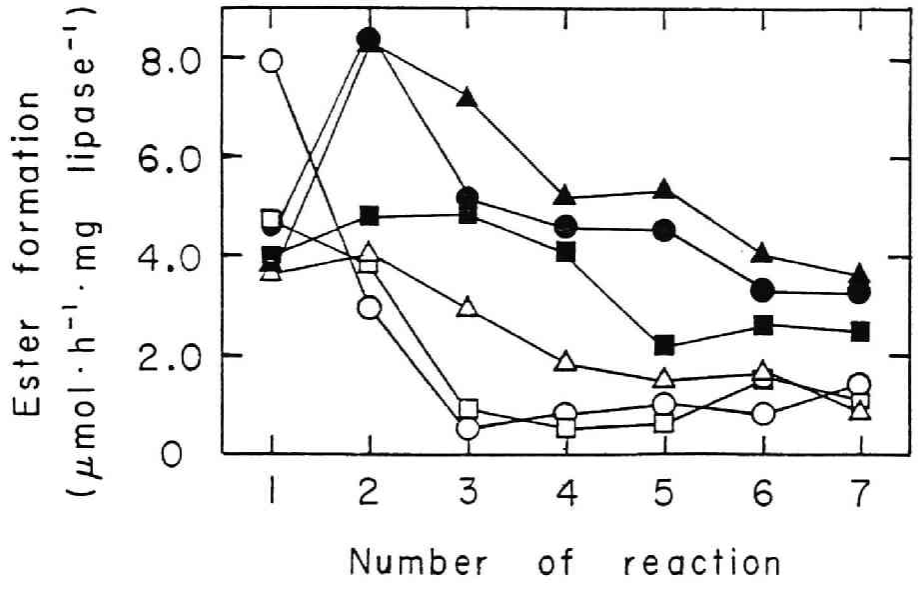


Fig. 3. Repeated use of lipase OF 360.

Each reaction was carried out for 22 h in water-saturated isooctane with C-lipase (○, △, □) or PU-3-C-lipase (●, ▲, ■) in the presence of 100 mM oleic acid as acyl donor at 40°C (○, ●), 30°C (△, ▲) or 20°C (□, ■), as described in the text.

SUMMARY

Fifty kinds of hydrolases were employed for screening of the enzymes having a high activity to esterify citronellol in an organic solvent. Although twenty-two hydrolases were excellently active as catalysts in the organic solvent, lipase from Candida cylindracea (lipase OF 360) was selected to examine the effects of reaction conditions on the enzyme activity based on the availability and the activity yield after immobilization. When the enzyme was entrapped in hydrophobic polyurethane gels, water-saturated isooctane was found to be the most suitable solvent, while polar solvents caused reversible inactivation. Entrapment significantly enhanced the operational stability of the lipase in the organic solvent.

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CHAPTER 2. Stereoselective Esterification of Halogen-Containing Carboxylic Acids by Lipases in Organic Solvents

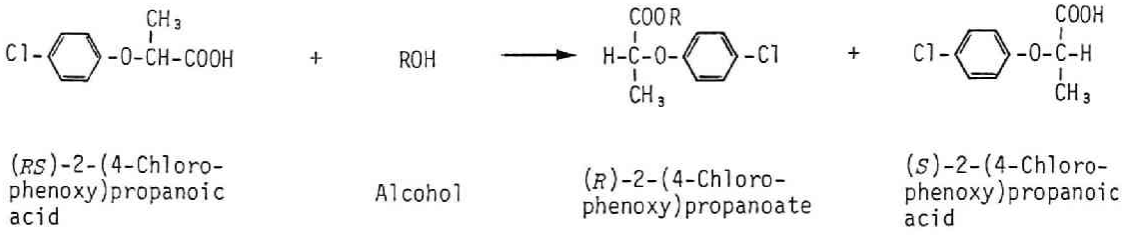
INTRODUCTION

The most attractive characteristic which makes biocatalysts far superior to conventional chemical catalysts is their high stereoselectivity, although the application of biocatalysts had been restricted to aqueous reaction systems. With the recent development of enzyme technology, however, especially in connection with immobilization techniques, biocatalysts have been applied not only in aqueous reaction systems but also in organic solvent reaction systems (1-3). Introduction of appropriate organic solvents to reaction systems can improve the poor solubility in aqueous solution of such substrates as lipophilic and water-insoluble compounds. Furthermore, when hydrolytic enzymes are applied in synthetic reactions, replacement of water with adequate organic solvent shift the reaction equilibrium toward synthetic direction. As one of the major

application of biocatalysts in organic solvents is the optical resolution of racemic compounds, stereoselective esterification in organic solvents has been developed (4-6).

The stereoselective esterification of menthol by polyurethane-entrapped yeast lipase was successfully carried out and chain length of acyl donors was found to affect remarkably the stereoselective esterification (5). Hitherto, most studies were mainly performed on the optical resolution of racemic alcohols and their esters, but systematic study concerning the stereoselective esterification of racemic carboxylic acids was very limited. In this chapter, the author has tried optical resolution of several racemic carboxylic acids through stereoselective esterification by hydrolases, which may have a potential of application in industrial fields. A combination of yeast lipase OF 360 and 2-(4-chlorophenoxy)propanoic acid was selected as the most suitable system to examine the effects of chain length of the second substrates, alcohols, on stereoselective esterification (Fig. 1). Selection of the second substrate

is important for optimization of reaction conditions, from the fundamental and practical viewpoints.



(*RS*)-2-(4-Chloro-phenoxy)propanoic acid | Alcohol | (*R*)-2-(4-Chloro-phenoxy)propanoate | (*S*)-2-(4-Chloro-phenoxy)propanoic acid

Fig. 1. Stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid catalyzed by yeast lipase in organic solvent.

MATERIALS AND METHODS

Materials

Lipase OF 360 from Candida cylindracea was the product of Meito Sangyo Co., Tokyo, Japan, and cholesterol esterase Type A from Pseudomonas sp. was a gift from Toyobo Co., Osaka, Japan. 2-(4-Chlorophenoxy)-propanoic acid was obtained from Aldrich Chemical Co.,

Milwaukee, WI, USA. Citronellol and 1-tetradecanol were purchased from Nacalai Tesque, Kyoto, Japan. Other chemicals were also obtained from commercial sources.

Adsorption of hydrolases on Celite

Enzyme powder (100 mg) was suspended in 100 µl of deionized water and then mixed thoroughly with 250 mg of Celite NO. 535.

Enzyme reactions

The reactions were carried out at 30°C with shaking (120 strokes.min^{-1}). The reaction mixtures were composed of Celite-adsorbed lipase OF 360 or Celite-adsorbed cholesterol esterase (corresponding to 100 mg enzyme powder) and 10 ml of water-saturated isooctane or benzene containing 100 mM alcohol and 100 mM acid.

Analysis

Alcohols and esters were determined by gas chromatography using a glass column (1 m) packed with silicon AN-600 or silicon OV-17 supported on chromosorb W AW DMCS (detector; FID). n-Heptadecane or n-icosane was used as

an internal standard.

Chromatographic separation

After esterification, the reactions were stopped by filtration of enzymes and addition of acetone and then solvents of the filtrates were removed by evaporation. 2-(4-Chlorophenoxy)propanoic acid and alcohols remained and the esters formed were isolated by column chromatography (Silica gel 60, 70 to 230 mesh). Eluent solvents used were n-hexane/ethyl acetate, 10:1 v/v for esters, n-hexane/ethyl acetate, 4:1 v/v for alcohols, and n-hexane/ethyl acetate/acetic acid, 20:5:1 v/v for 2-(4-chlorophenoxy)-propanoic acid.

Hydrolysis of esters formed

1-Tetradecyl 2-(4-chlorophenoxy)propanoate purified from the reaction mixture by column chromatography was hydrolyzed with 0.4 N NaOH in methanol at 80°C for 10 min. Hydrolyzed mixture was evaporated to remove methanol and extracted with ethyl ether after addition of 0.1 N HCl to neutralize. 2-(4-Chlorophenoxy)propanoic acid obtained by

hydrolysis of the ester was isolated from the hydrolyzed mixture by column chromatography.

Measurement of optical rotation

The optical rotation of acids and esters was measured at 589 nm in ethanol with a DIP-140 type polarimeter (Japan Spectroscopic Co., Tokyo, Japan). The specific rotation $[\alpha]$ was estimated using the following equation: $[\alpha] = a/(C \times l)$, where a is the observed rotation; C, concentration of the compounds (g.100 ml^{-1}) and l, light path (mm).

Analysis with HPLC

Enantiomeric excess of 2-bromohexanoic acid and 3-chlorobutanoic acid was determined by HPLC equipped with a model 6000A instrument (Waters, Milfold, MA, USA) and a differential refractometer R401 (Waters) using a chiralcel OB column (i.d. = 0.46 cm; l = 25 cm; Daicel Chemical Industries, Tokyo, Japan) (eluent: 1-hexanol/2-propanol, 19:1 v/v; flow rate: 0.5 $ml.min^{-1}$). Under these conditions, the enantiomers of 2-bromohexanoic acid and 3-chlorobutanoic acid could be separated. Enantiomeric excess

(% ee) was calculated by using the following equation (($\underline{l}$ - $\underline{d}$)/($\underline{l}$ + $\underline{d}$)) x 100, where $\underline{l}$ and $\underline{d}$ were the peak area of ($\underline{S}$)- and ($\underline{R}$)-enantiomers of the acids obtained by analysis with HPLC. The enantiomers of 2-(4-chlorophenoxy)propanoic acid were also separated successfully by HPLC using the same chiralcel column (eluent: 1-hexanol/2-propanol, 9:1 v/v; flow rate: 0.4 $ml.min^{-1}$). Enantiomeric excess of this acid determined by HPLC was comparable to that obtained by measurement of its optical rotation.

RESULTS AND DISCUSSION

The author has already described that many hydrolases can actively catalyze the synthesis of an ester in organic solvent (7). Among these hydrolases, lipase OF 360 and cholesterol esterase Type A were selected because they have been found to exhibit high esterification activity in organic solvent and are relatively inexpensive.

Substrate specificity and stereoselectivity of lipase OF 360 and cholesterol esterase Type A for carboxylic acids, especially those containing halogen atom, have been studied with racemic citronellol as the second substrate because

halogen-containing acids may be useful as chiral synthons. As shown in Table 1, the substrate specificity and stereoselectivity of lipase OF 360 for carboxylic acids as acyl donors were different from those of cholesterol esterase Type A. Among the acids tested with lipase OF 360, 3-chlorobutanoic acid was converted stereoselectively with a relatively low activity. 2-Bromohexanoic acid was esterified with a high activity but without stereoselectivity. In the meantime, it was found that lipase OF 360 catalyzed the esterification of 2-(4-chlorophenoxy)propanoic acid with both high activity and high stereoselectivity. In the case of cholesterol esterase Type A, the esterification activity for most of the acids examined was lower than that of lipase OF 360, and 2-(4-chlorophenoxy)propanoic acid was not converted to the ester stereoselectively. Citronellol was used as the acyl acceptor by both the enzymes without stereoselectivity under the conditions employed. From these results, the author selected the esterification of 2-(4-chlorophenoxy)propanoic acid by lipase OF 360 as a suitable model system to examine the effect of the chain length of alcohols on

Table 1. Substrate specificity and stereoselectivity of lipase OF 360 and cholesterol esterase Type A[a]

Acid	Lipase OF 360		Cholesterol esterase	
	Relative activity[b]	% ee[c]	Relative activity[b]	% ee[c]
2-Bromopropanoic	nil	-	3.5	-
2-Chloropropanoic	5.6	-	6.7	-
2-Bromobutanoic	-	-	0.8	-
3-Chlorobutanoic	14.9	49.7	2.2	-
2-Chlorobutanoic	1.8	-	4.8	-
2-Phenylpropanoic	13.4	-	79.6	-
2-Phenylbutanoic	nil	-	23.9	-
2-Bromohexanoic	184	0.3	1.0	-
2-(4-Chlorophenoxy)-propanoic	100	52.0	12.4	nil

(Footnote of Table 1)

[a] The reactions were carried out with racemic citronellol in water-saturated isooctane except for the esterification of 2-(4-chlorophenoxy)propanoic acid in water-saturated benzene.

[b] Ester formation rate with 2-(4-chlorophenoxy)propanoic acid by lipase OF 360 was expressed as 100.

[c] Enantiomeric excess of the respective remaining acids. In the case of 3-chlorobutanoic acid and 2-bromohexanoic acid, the enantiomeric excess was determined by HPLC at 39 % conversion and 54 % conversion, respectively. Specific rotation ($\underline{C}$ = 1, ethanol) of remaining 3-chlorobutanoic acid was +17.1. In the case of 2-(4-chlorophenoxy)propanoic acid, enantiomeric excess was determined at 42 % conversion for lipase OF 360 and 55 % conversion for cholesterol esterase from the specific rotation compared with the value of $[\alpha]_D^{25}$ = -40.1 ($\underline{C}$ = 1, ethanol) obtained from Bailstein Handbuch (Beilstein-Institut, Frankfurt, Germany).

stereoselective esterification. Furthermore, (R)-enantiomer (d-isomer) of 2-(4-chlorophenoxy)propanoic acid is an important herbicide, and optical resolution of this acid will be of importance for industrial application.

The effect of chain length of fatty acids used as acyl donors on stereoselective esterification of menthol catalyzed by lipase OF 360 was already investigated (5). It was found that fatty acids having longer chain served as excellent acyl donors for the esterification, while shorter chain fatty acids were not good substrates. On the contrary, sufficient stereoselectivity for menthol was not observed with longer chain fatty acids in contrast to the high stereoselectivity with shorter chain acids. Therefore, it will be of interest to investigate the effect of chain length of alcohols on stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid.

Esterification of 2-(4-chlorophenoxy)propanoic acid with six kinds of alcohols having different chain length was followed along with the reaction time. As shown in Fig. 2, the reaction rate with shorter chain alcohols such as ethanol was high and the reaction continued exceeding 50 %

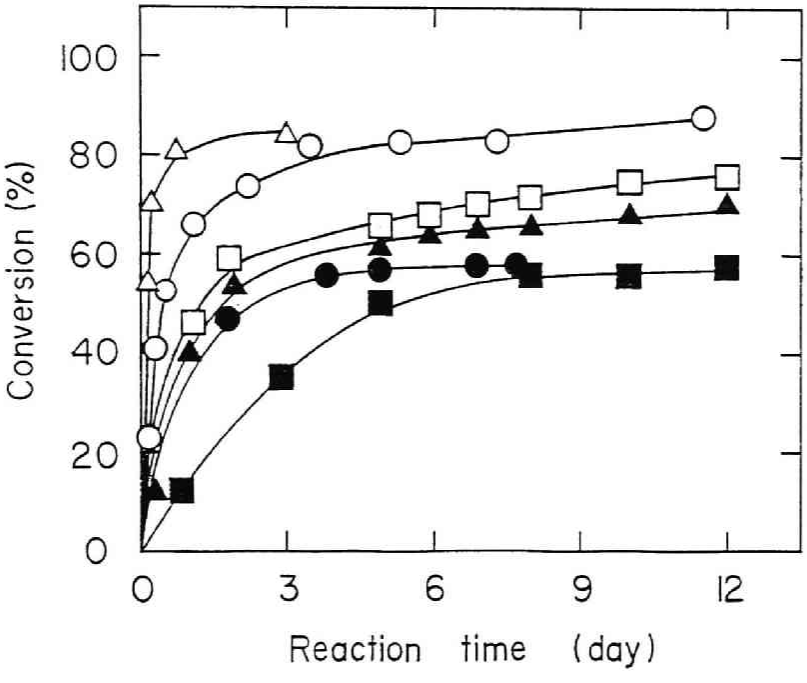


Fig. 2. Time-course of esterification of 2-(4-chlorophenoxy)propanoic acid with different alcohols by lipase OF 360. (△), Ethanol; (○), 1-hexanol; (□), 1-nonanol; (▲), 1-decanol; (●), 1-tetradecanol; (■), 1-octadecanol.

conversion, while the esterification with longer chain alcohols such as 1-octadecanol proceeded slowly and almost stopped after the conversion reached around 50 %. These results suggested the high stereoselectivity with longer chain alcohols and the low stereoselectivity in the case of shorter chain alcohols.

The results in Table 2 clearly demonstrate that chain length of alcohols seriously affects the ester formation rate and the stereoselectivity judged from the optical purity of the remaining acid. When the esterification was stopped at nearly 50 % conversion, it was found that longer chain alcohols took much more time to reach that value than shorter chain alcohols. On the other hand, the longer the chain length of alcohols was, the higher the stereoselectivity was observed, so far as the enantiomeric excess of the remaining acid was examined. These phenomena showed striking contrast to the results on the effects of the chain length of acyl donors on menthol esterification (5). These results are of great interest in both the basic research and industrial application.

Therefore, a relationship between optical purity of the

Table 2. Effect of chain length of alcohols on stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid by lipase OF 360[a]

Alcohol	Reaction time (h)	Conversion (%)	$[\alpha]_D^{25}$ (deg)[b]	% ee[c]
Methanol	3	53.5	-3.76	8.2
Ethanol	3	54.5	-7.16	17.9
1-Propanol	4	53.5	-8.35	20.8
1-Butanol	5	52.0	-17.30	43.1
1-Hexanol	14	52.1	-25.30	63.0
1-Heptanol	25	53.0	-27.60	68.8
1-Nonanol	31	52.0	-30.20	75.3
1-Decanol	46	54.0	-31.40	78.3
1-Tetradecanol	72	55.0	-33.00	82.3
1-Octadecanol	216	52.0	-33.20	82.8

[a] The reactions were carried out in water-saturated benzene.

[b] Specific rotation of the remaining acid.

[c] Enantiomeric excess of the remaining acid was determined as described in Table 1.

remaining acid and conversion ratio was studied by using three typical alcohols with different chain length, that is, ethanol, 1-hexanol and 1-tetradecanol. As illustrated in Fig. 3, optical purity of the remaining acid increased with increase in the degree of conversion. In the case of ethanol, enantiomeric excess of the remaining acid was only 30 % even when the conversion reached about 70 %. In the case of 1-hexanol, enantiomeric excess of the remaining acid reached 85 %, but a yield of the acid was low because this value was obtained at 70 % conversion of the acid. In the meantime, 1-tetradecanol gave a high yield of the highly optically pure acid by stereoselective esterification. Namely, enantiomeric excess of the remaining acid was more than 98 % at 59 % conversion. These results indicate that 1-tetradecanol, which is a long chain alcohol, is most suitable among the alcohols tested for optical resolution of 2-(4-chlorophenoxy)propanoic acid, while short or medium chain alcohols are not good for this purpose. More longer chain alcohols than 1-tetradecanol, such as 1-octadecanol, may be better for the highly stereoselective esterification, but, from industrial viewpoints, it is not

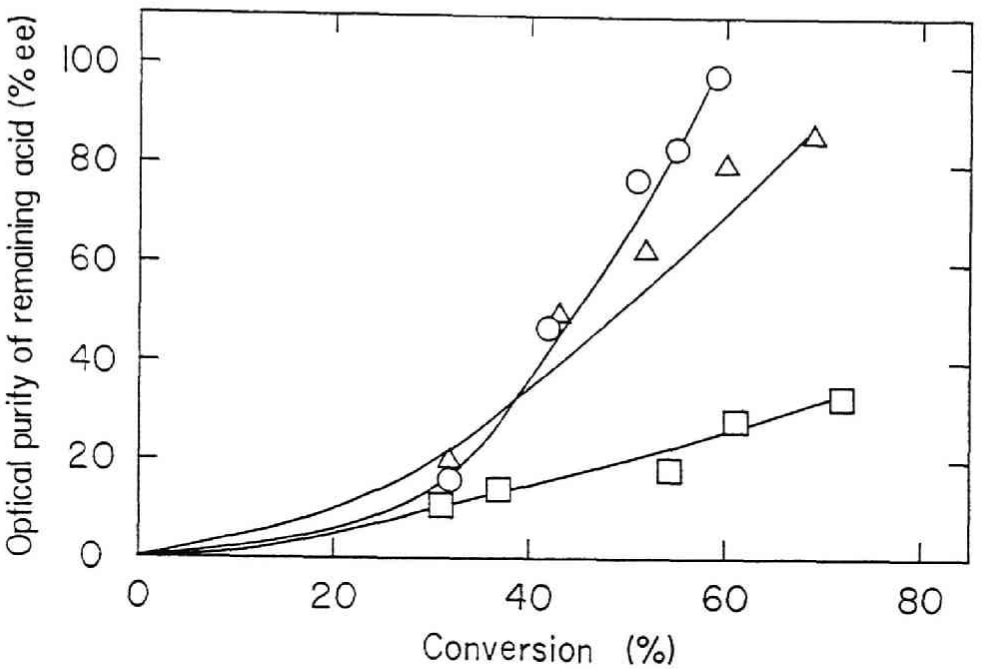


Fig. 3. Relationship between optical purity of the remaining acid and conversion ratio in esterification of 2-(4-chlorophenoxy)propanoic acid with different alcohols by lipase OF 360. (○), 1-Tetradecanol; (△), 1-hexanol; (□), ethanol.

economic because the reaction rate with 1-octadecanol was much lower than that with 1-tetradecanol (Table 2).

Amounts of (R)- and (S)-enantiomers of 2-(4-chlorophenoxy)propanoic acid converted were calculated respectively based on enantiomeric excess of the remaining acid and conversion ratio shown in Fig. 3. The results shown in Fig. 4 clearly demonstrated that, in the case of 1-tetradecanol, lipase OF 360 catalyzed preferentially the esterification of (R)-enantiomer, thus a typical enzyme-catalyzed kinetic resolution of the racemic acid being possible. In contrast, ethanol served as a good substrate for the esterification of both (R)- and (S)-enantiomers of the acid, showing a low stereoselectivity.

The author also examined the optical purity of 1-tetradecyl 2-(4-chlorophenoxy)propanoate formed with the progress of the reaction (Fig. 5). The enantiomeric excess of the ester formed was high and almost constant below about 50 % conversion. These results were consistent with those of the optical purity observed with the remaining acid, indicating that 2-(4-chlorophenoxy)propanoic acid was esterified stereoselectively with 1-tetradecanol by lipase

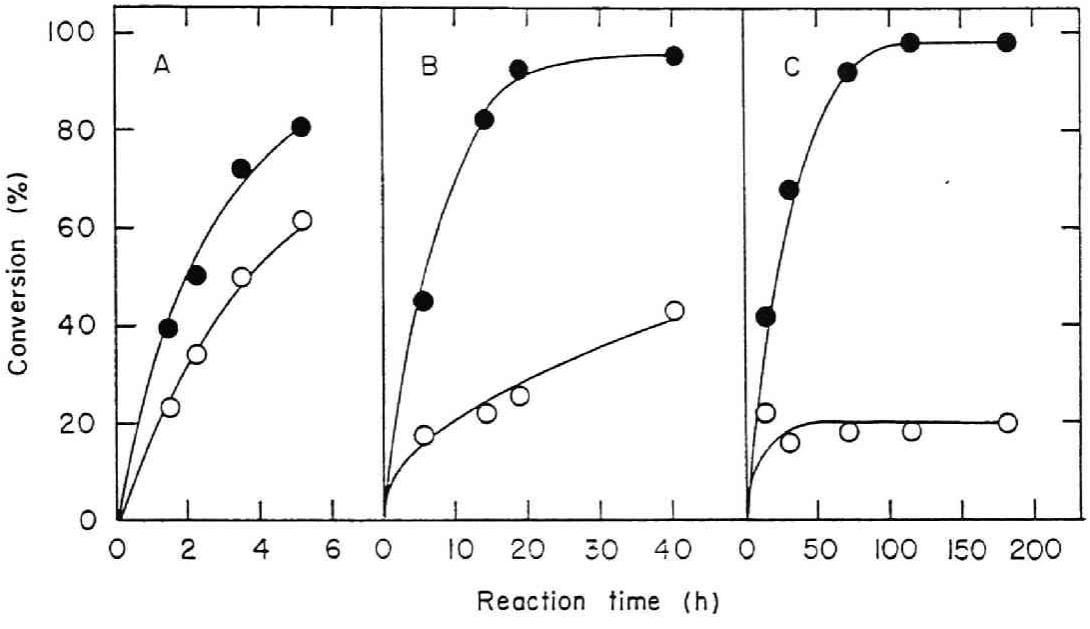


Fig. 4. Stereoselectivity of esterification of 2-(4-chlorophenoxy)propanoic acid with different alcohols by lipase OF 360. A, ethanol; B, 1-hexanol; C, 1-tetradecanol. (○), (S)-enantiomer of the acid; (●), (R)-enantiomer of the acid.

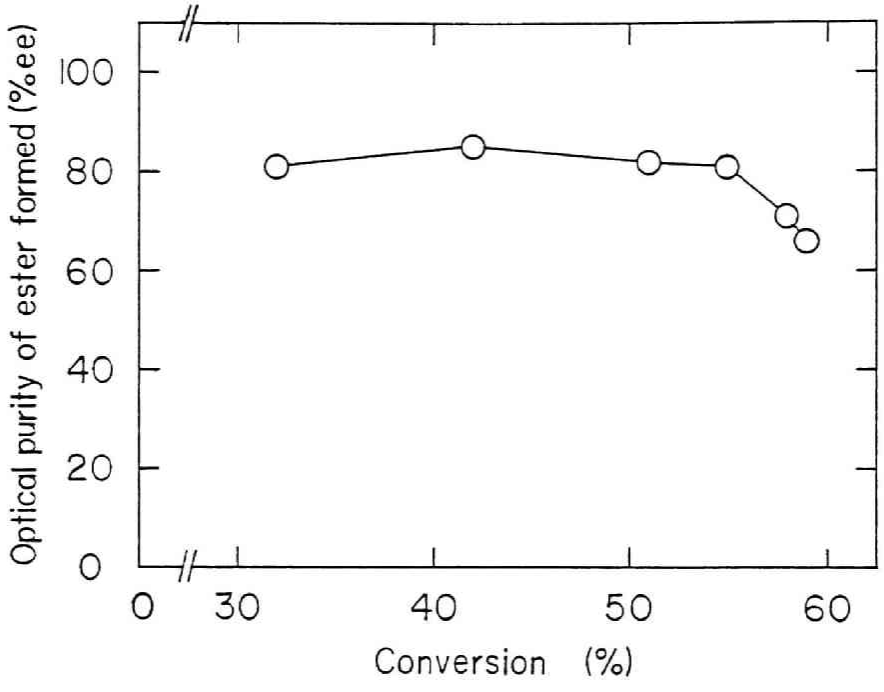


Fig. 5. Optical purity of the ester formed in esterification of 2-(4-chlorophenoxy)propanoic acid with 1-tetradecanol by lipase OF 360. Enantiomeric excess of the ester formed was obtained as follows: The ester formed was hydrolyzed chemically and optical rotation of 2-(4-chlorophenoxy)propanoic acid resulted was measured after purified by column chromatography.

OF 360.

From the results mentioned above, the author can conclude that chain length of alcohols is a very important factor for stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid by lipase OF 360. The longer chain length of alcohols was, the higher the stereoselectivity was achieved. Lipase OF 360 catalyzed the esterification with 1-tetradecanol in a highly stereoselective manner with a moderate reaction rate. Optically active 2-(4-chlorophenoxy)propanoic acid and its ester were obtained with high yields by using Celite-adsorbed yeast lipase in an organic solvent. Furthermore, the results obtained here give a clue to image the action mode of lipase.

It is said that an acyl donor attacks lipase and forms an acyl-enzyme, then an alcohol reacts with the acyl-enzyme to yield an ester. If the results obtained in this work is explained on the basis of this concept, the reaction rate should be controlled at the step of deacylation of the acyl-enzyme by the steric factor of alcohol to be the second substrate. In the case of longer chain alcohols, the

reaction rate will be low probably due to their steric hindrance. Stereoselectivity must be due to the difference in the reactivity of the alcohols toward the acyl-enzymes formed from (R)- and (S)-2-(4-chlorophenoxy)propanoic acid. The fact that the longer chain alcohols, which have higher steric hindrance, are more effective substrates for highly stereoselective esterification than the shorter chain ones may be explained by considering such the difference.

SUMMARY

Optical resolution of racemic carboxylic acids containing halogen atom was tried with stereoselective esterification by Celite-adsorbed hydrolases in organic solvents. As lipase OF 360 from Candida cylindracea was found to esterify 2-(4-chlorophenoxy)propanoic acid stereoselectively, whose (R)-enantiomer (d-isomer) is an important herbicide, the effect of chain length of alcohols on the stereoselectivity as well as the reaction rate have been investigated. The results revealed that chain length of alcohols seriously affected the stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid, that is, long chain alcohols, such as 1-tetradecanol, served as the excellent substrates for the optical resolution of the acid, although the reaction rate was moderate.

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CHAPTER 3. Long-Term Continuous Production of Optically Active 2-(4-Chlorophenoxy)propanoic Acid by Celite-Adsorbed Yeast Lipase in Organic Solvent Systems

INTRODUCTION

From the industrial viewpoints, it is one of the most important subjects to construct continuously operated biochemical systems for production of useful substances. In application of immobilized enzymes and immobilized microbial cells in aqueous systems, many examples of continuously operated bioreactors have been reported (1-4).

For the bioconversion of various lipophilic or water-insoluble compounds, it is essential to introduce organic solvents into reaction systems to improve the solubility of these reactants. Furthermore, organic solvents can shift the reaction equilibrium to synthetic direction in the case of hydrolytic enzymes by reducing the water fraction in the systems (5-7).

The author's laboratory has demonstrated bioconversion

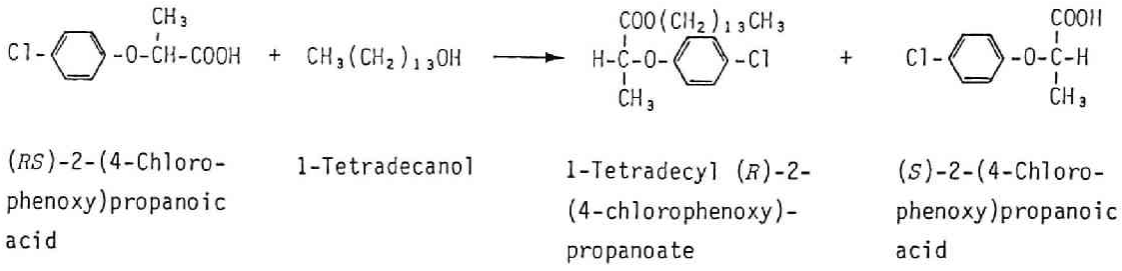

Fig. 1. Stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid with 1-tetradecanol by lipase OF 360.

of various lipophilic compounds in organic solvent systems (8-9), such as optical resolution of menthol (10) and 2-(4-chlorophenoxy)propanoic acid (11) by the stereoselective esterification with lipase. In spite of many works on bioconversion in organic solvents, there have so far been only a few reports on continuous bioconversion (12-15) and no study on a continuously operated system applying stereoselectivity of biocatalysts.

This chapter deals with long-term continuous production of optically active 2-(4-chlorophenoxy)propanoic acid, (R)-isomer of which is an important herbicide, by the stereoselective esterification with 1-tetradecanol by yeast

lipase in organic solvents (Fig. 1), and with the optimization of the operation conditions.

MATERIALS AND METHODS

Materials

Lipase OF 360 from Candida cylindracea was purchased from Meito Sangyo Co., Tokyo, Japan. (RS)-2-(4-Chlorophenoxy)propanoic acid was a product of Aldrich Chemical Co., Milwaukee, WI, USA, and 1-tetradecanol was obtained from Nacalai Tesque, Kyoto, Japan. Celites (No.535, No.545 and Hyflo Super Cel) were products of Johnes-Manville Co., Denver, CO, USA. All other chemicals were also obtained from commercial sources.

Adsorption of lipase on Celite

Unless stated otherwise, 100 mg of lipase OF 360 was suspended in 100 µl of deionized water and then mixed thoroughly with 250 mg of Celite No. 535.

Batchwise enzymatic reactions

The stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid with 1-tetradecanol by lipase was carried out at 30°C in a long-necked flask with shaking (120 strokes.min^{-1}). The reaction mixtures were composed of Celite No. 535-adsorbed lipase OF 360 (corresponding to 100 mg of lipase) and 10 ml of water-saturated organic solvent containing 100 mM 2-(4-chlorophenoxy)propanoic acid and 100 mM 1-tetradecanol.

Continuously operated enzymatic reactions

Continuous optical resolution of 2-(4-chlorophenoxy)propanoic acid by lipase-catalyzed esterification with 1-tetradecanol was carried out in a packed-bed column reactor (PBR). Celite-adsorbed lipase OF 360 (corresponding to 10 g of lipase) was packed in a glass column (2 x 30 cm) and temperature of the reactor was maintained at 30°C. Water-saturated organic solvent containing 100 mM 2-(4-chlorophenoxy)propanoic acid and 100 mM 1-tetradecanol was supplied continuously from top of the PBR to bottom (down-flow-type PBR) or from bottom to top using a pump (type L-6000, Hitachi Co., Tokyo, Japan) (up-

flow-type PBR).

Preservation of PBR

After cessation of supply of substrate solution, the temperature of PBR was maintained at 4°C.

Treatment of PBR

Acetone (500 ml) containing 100 mM of the substrates was supplied from top of the PBR to bottom at a flow rate of 8.0 $l.day^{-1}$ for 1.5 h.

Residual activity of Celite-adsorbed lipase after continuous operation

Celite-adsorbed lipase OF 360 which was taken out from the some parts of PBR after continuous operation was washed with water-saturated organic solvent, and the organic solvent was removed under vacuum. Celite-adsorbed lipase obtained after removal of the solvent (450 mg) was used for batchwise reaction as mentioned in "Batchwise enzymatic reactions" and the initial ester formation rate was measured to determined its residual activity. Initial

activity of Celite-adsorbed lipase was used as 100% to calculate the residual activity.

Content of lipase on Celite

Celite-adsorbed lipase OF 360 was washed with acetone and dried. Lipase OF 360 was extracted with 1 N NaOH and determined by Biuret method (16).

Analysis

The remaining alcohol and the ester formed were determined by gas chromatography using a glass column (1.1 m) packed with silicon OV-17 supported on chromosorb W AW DMCS (carrier gas, N_2; flow rate, 60 ml.min^{-1}; injector temperature, 300°C) by using n-icosane as an internal standard. With the use of temperature programs from 140°C to 160°C at 4°C.min^{-1} and from 160°C to 280°C at 20°C.min^{-1}, the retention times observed were 4.5 min for 1-tetradecanol, 5.4 min for 2-(4-chlorophenoxy)propanoic acid, 7.2 min for n-icosane, and 11.6 min for 1-tetradecyl 2-(4-chlorophenoxy)propanoate. Optical purity of the remaining (S)-acid was determined from optical rotation, measured with a DIP-140 type polarimeter (Japan Spectroscopic Co., Tokyo,

Japan), as described in CHAPTER 2 (11).

RESULTS AND DISCUSSION

Effect of organic solvents in batchwise reactions

To construct a continuously operated system having high productivity, it is essential to select an adequate organic solvent because the hydrophobicity of the organic solvent seriously affects the activity of lipase OF 360 (10, 17). Water-miscible or hydrophilic organic solvents tend to inactivate enzymes, while hydrophobic solvents are known to be favourable for enzymes. In particular, isooctane and cyclohexane are excellent solvents for the enzymatic reactions catalyzed by yeast lipase, but 2-(4-chlorophenoxy)propanoic acid is insoluble in these solvents. Therefore, the author tested other kinds of hydrophobic solvents, in which the substrates are soluble, on the stereoselective esterification with lipase OF 360 in a batchwise reaction (Table 1).

As for the activity of lipase OF 360, carbon tetrachloride-isooctane (8:2, v/v) gave an excellent result,

Table 1. Effect of organic solvents on stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid by Celite-adsorbed lipase OF 360 in batchwise reaction

Organic solvent	Relative reaction rate[a]			% ee[b]
	1st batch	2nd batch	2nd/1st	
Benzene	100	95	95	77.2
Toluene	198	163	82	-
o-Xylene	334	194	58	-
1,2-Dichloroethane	130	-	-	-
Carbon tetrachloride	387	236	61	-
Methyl isobutyl ketone	29	-	-	-
Isopropyl ether	112	-	-	-
Benzene - n-hexane (7:3, v/v)	114	-	-	-
Benzene - cyclohexane (7:3, v/v)	117	-	-	-
Benzene - isooctane (7:3, v/v)	144	-	-	-
Carbon tetrachloride - isooctane (8:2, v/v)	460	283	61	75.8

(Footnote of Table 1)

All solvents were saturated with water. Batchwise reaction was carried out for 56 h.

[a] Ester formation rate in benzene was expressed as 100.

[b] Enantiomeric excess of the remaining acid was determined at 51 % conversion in 1st batchwise reaction.

but the stability of lipase was moderate. On the contrary, benzene gave a moderate result for the lipase activity, but the stability was excellent. Similar optical purity of the remaining (S)-2-(4-chlorophenoxy)propanoic acid was observed in both solvents. Consequently, the author selected both solvent systems to examine the continuous production.

Effect of water content in adsorbed lipase

It is well known that water seriously affects the activity of enzymes in organic solvents (17-18). Furthermore, water, which is another product of the esterification reaction, is presumed to accumulate in the reactor and inhibit the reaction in the case of long-term continuous operation. Therefore, the effect of water content in the Celite-adsorbed lipases on the esterification of 2-(4-chlorophenoxy)propanoic acid in an organic solvent system was investigated in batchwise reaction.

As shown in Fig. 2, lipase maintained high activity in a relatively wide range of water content (1.0 - 2.0 μl.mg lipase^{-1}), although the optimum water content (1.5 μl.mg lipase^{-1}) was observed. From the results obtained, the

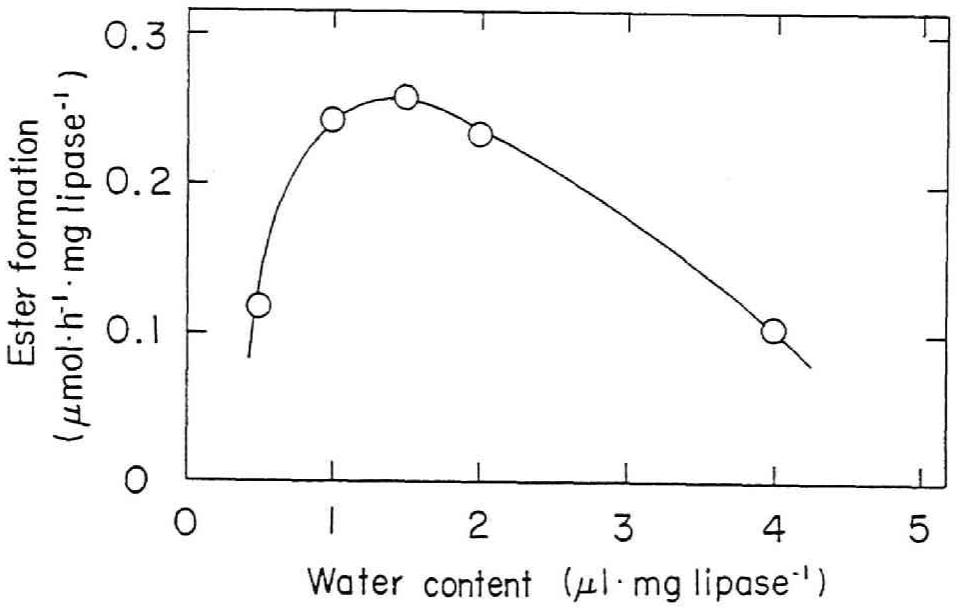

Fig. 2. Effect of water content in Celite-adsorbed lipase OF 360 on stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid in batchwise reaction. Water content was controlled by the amount of deionized water used to suspend 100 mg of lipase OF 360. The reactions were carried out in water-saturated carbon tetrachloride-isooctane (8:2, v/v).

author chose 1.0 μl.mg lipase^{-1} as water content to prepare the Celite-adsorbed enzyme, considering the water to be accumulated in the course of the continuous esterification.

Effect of organic solvents on continuous production of optically active 2-(4-chlorophenoxy)propanoic acid

Based on the results mentioned above, continuous production of optically active 2-(4-chlorophenoxy)-propanoic acid by stereoselective esterification with Celite-adsorbed lipase OF 360 was tried in a packed-bed column reactor (PBR) supplied with the substrate solution from top to bottom. It is essential to maintain the conversion ratio at 50 % with high stereoselectivity and a large flow rate for a long period to achieve the efficient continuous optical resolution. As mentioned above, carbon tetrachloride-isooctane (8:2, v/v) was superior to other solvents as for the ester formation rate, while benzene would be better for lipase stability. In the case of PBR using benzene-isooctane (7:3, v/v), the conversion dropped in a short period at two different flow rates, 144 ml.day^{-1} and 72.0 ml.day^{-1} (Fig. 3A and B). However, the

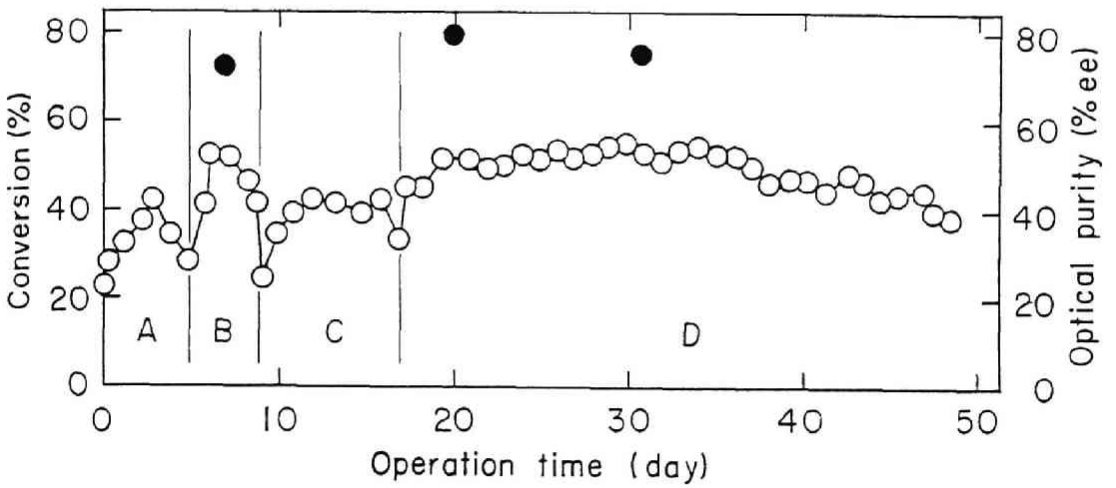

Fig. 3. Effect of organic solvents and flow rates on continuous stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid in a column reactor packed with Celite-adsorbed lipase OF 360 (PBR). Water-saturated benzene-isooctane (7:3, v/v) (A and B) or water-saturated carbon tetrachloride-isooctane (8:2, v/v) (C and D) was examined as reaction solvents. The substrate solution was supplied from top of the PBR with a flow rate of (A) 144 $ml.day^{-1}$, (B) 72.0 $ml.day^{-1}$, (C) 173 $ml.day^{-1}$ or (D) 86.4 $ml.day^{-1}$. (○), Conversion (%); (●), optical purity of the remaining acid (% ee).

conversion ratio at approximately 50 % could be kept with a flow rate of 86.4 ml.day^{-1} for over 17 days and highly optically active 2-(4-chlorophenoxy)propanoic acid was produced continuously when water-saturated carbon tetrachloride-isooctane (8:2, v/v) was employed (Fig. 3D), although the conversion ratio was lower than 50 % with a flow rate of 173 ml.day^{-1} (Fig. 3C). Therefore, water-saturated carbon tetrachloride-isooctane (8:2, v/v) was selected as a reaction solvent in the subsequent continuous operation.

Effect of particle size of supports and style of flow in PBR

In PBR, it is thought that particle size of supports affects the productivity, thus three kinds of Celites, which have almost the same composition but different particle size, Hyflo Super Cel (18 μm as median particle size), Celite No. 535 (25 μm) and Celite No. 545 (26 μm), were used for the preparation of the adsorbed-lipase. Conversion ratios with three preparations became constant at nearly 40 % in all cases and remarkable difference was

not observed so far as the flow rate of the substrate solution was adjusted to 173 ml.day^{-1} (Fig. 4).

Furthermore, the difference in the productivity between up-flow- and down-flow-type PBR was examined at a flow rate of 173 ml.day^{-1}. However, there was no remarkable difference in both types. These results indicated that the particle size of supports and the style of flow did not so seriously affect the productivity under the conditions employed.

Long-term continuous production of optically active 2-(4-chlorophenoxy)propanoic acid

Based on the results obtained, a down-flow-type PBR packed with Celite-adsorbed lipase was employed because of the simplicity of the system for long-term continuous operation in the production of optically active 2-(4-chlorophenoxy)propanoic acid in an organic solvent system (Fig. 5). Under the optimized conditions, that is, water-saturated carbon tetrachloride-isooctane (8:2, v/v) as a reaction solvent, Celite No. 535 as a support and a flow rate of 86.4 ml.day^{-1}, the reactor could be successfully operated by keeping about 50 % conversion for 34 days. It

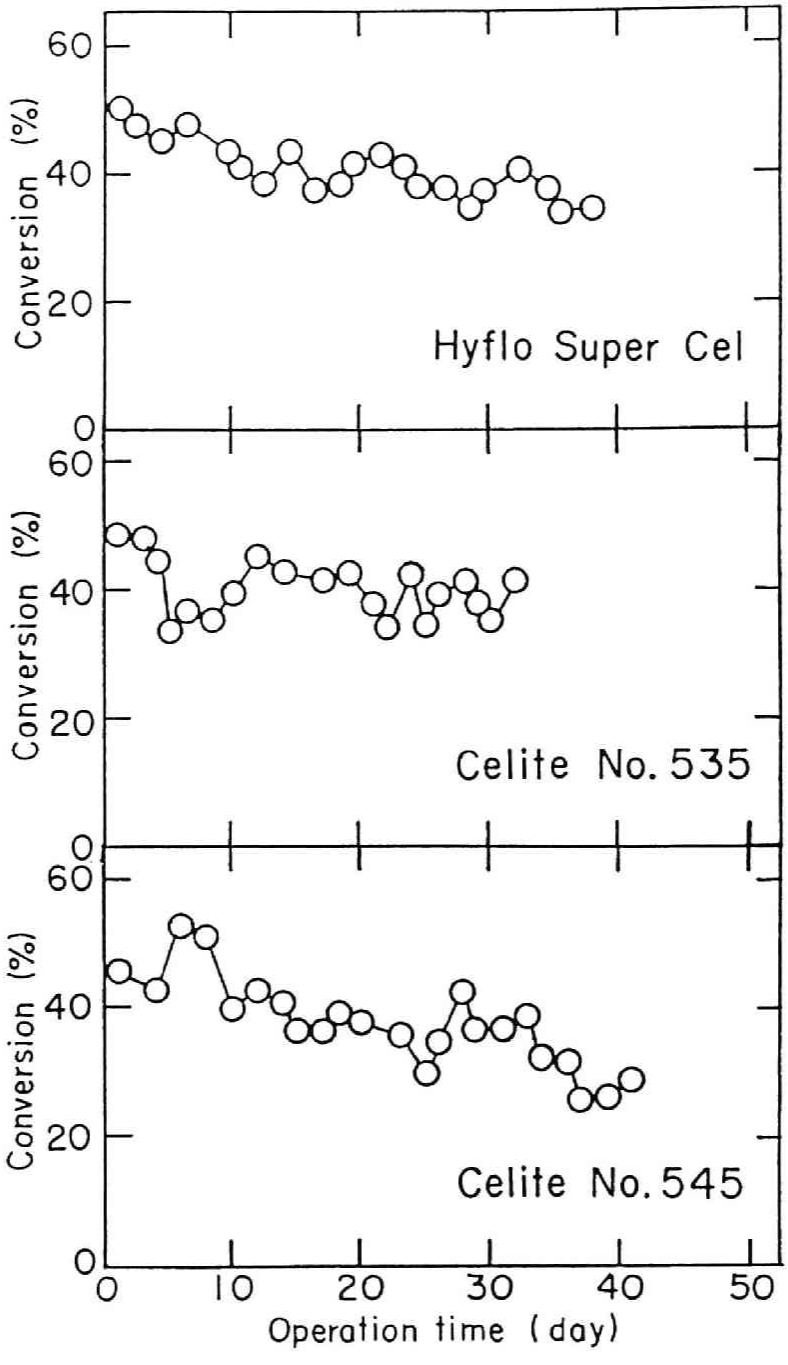
Conversion (%)
60
40
20
0
Hyflo Super Cel
Celite No. 535
Celite No. 545
0
10
20
30
40
50
Operation time (day)

Fig. 4. Effect of particle size of supports on continuous stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid by adsorbed lipase OF 360 in an organic solvent system. Water-saturated carbon tetrachloride-isooctane (8:2, v/v) containing each 100 mM of the substrates was supplied continuously from the top of the PBR with a flow rate of 173 $ml.day^{-1}$. The median particle size of Hyflo Super Cel, Celite No. 535, and Celite No. 545 was 18 μm, 25 μm, and 26 μm, respectively.

Conversion (%)
Optical purity (%ee)
Operation time (day)
0
20
40
60
80
100
0
50
100
150
200

Fig. 5. Long-term continuous stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid by Celite-adsorbed lipase OF 360 in an organic solvent system. Water-saturated carbon tetrachloride-isooctane (8:2, v/v) containing each 100 mM of the substrates was supplied from the top of the column reactor packed with Celite No. 535-adsorbed lipase OF 360 with a flow rate of 86.4 ml.day^{-1}. Broken line; preservation of the reactor at 4 ^{o}C. Arrows; treatment of the reactor with acetone containing each 100 mM of substrates. (○), Conversion (%); (●), optical purity of the remaining acid (% ee).

was also indicated that preservation of PBR at 4°C for 6 days was possible. However, the conversion ratio decreased less than 50 % after 34 days, probably due to the accumulation of water in the reactor, which is another product of esterification reaction. Hydrophilic organic solvents are generally used to remove water from immobilized biocatalysts. Therefore, the treatment of the reactor with acetone was carried out. As shown in Fig. 5, the lipase activity was recovered by the acetone treatment with keeping the conversion ratio above 50 % for further 29 days, although the second treatment with acetone was not effective. Such the long operation of an enzyme reactor in an organic solvent system has never been reported. No appreciable reaction was observed in a reactor packed with only Celite No. 535 in the absence of lipase, the results indicating that lipase OF 360 was surely active for a long period in the organic solvent. The stereoselectivity of the continuous reaction toward (R)-2-(4-chlorophenoxy)propanoic acid was as high as or higher than that of the batchwise reaction. In practice, the highest optical purity of the remaining acid in continuous reaction reached 95 % ee.

Furthermore, the conditions of PBR after the long-term continuous operation were investigated. The reaction solution and the Celite-adsorbed lipase were taken out from the PBR and the ester formed in the reactor, the content of lipase on Celite, and the residual activity of Celite-adsorbed lipase were measured. It was confirmed that the degree of conversion of 2-(4-chlorophenoxy)propanoic acid had become greater along with the flow of the substrate solution through the reactor (Fig. 6). The content of lipase on Celite was constant at all parts of the reactor (Fig. 6), and it could be said that lipase was effectively adsorbed on Celite and was not washed out by the continuous flow of the substrate solution. Furthermore, the residual activity of Celite-adsorbed lipase was measured after the continuous operation. Celite-adsorbed lipase, which was near the top of the bed (6 cm and 12 cm), was significantly inactivated. On the contrary, the enzyme far from the top (18 cm) retained the activity (Fig. 6). These results showed that inactivation of the lipase in the PBR proceeded from the top of the bed to bottom in the case of the down-flow-type. Then, the activity of the reactor will be able to be restored efficiently by the

Content of lipase on Celite
(mg lipase·mg Celite-lipase⁻¹)
0.4
0.2
0
Residual activity (%)
60
40
20
0
Ester formed in reactor (%)
60
40
20
0
0
6
12
18
24
Length (cm)
0 cm
6 cm
12 cm
18 cm
24 cm
Packed-bed column reactor
(φ2cm X 30cm)
Operation time , 195 days

Fig. 6. Conditions of the PBR after long-term continuous operation. The continuous operation was carried out as shown in Fig. 5 for 195 days. The internal reaction solution and the Celite-adsorbed lipase were took out from the PBR at the indicated length from top of the bed, and determined the residual activity (○), the content of lipase on Celite (●), and the ester formed in reactor (△). The ester formed in reactor was expressed in the form of conversion ratio (%).

replacement of only upper part of the bed.

In this study, the author could produce highly optically active 2-(4-chlorophenoxy)propanoic acid continuously for 63 days by Celite-adsorbed lipase OF 360 in water-saturated carbon tetrachloride-isooctane (8:2, v/v) as a reaction solvent, the results revealing that the lipase in the reactor exhibited its catalytic activity and stereoselectivity for more than 6 months in the organic solvent.

SUMMARY

Long-term continuous optical resolution of 2-(4-chlorophenoxy)propanoic acid was carried out by stereoselective esterification with Celite-adsorbed lipase OF 360 from Candida cylindracea by using 1-tetradecanol as the second substrate in an organic solvent system. Water content in Celite-adsorbed lipase affected the productivity, 1.0 μl.mg lipase^{-1} being most adequate for preparation of adsorbed lipase. Water-saturated carbon tetrachloride-isooctane (8:2, v/v) was found to be an excellent organic solvent for the continuous operation, while the change of particle size of Celites did not exert any effect on the productivity. Under the optimized conditions, (R)-enantiomer of the acid was continuously esterified with a high stereoselectivity in a packed-bed column reactor for 34 days. Furthermore, it was found that the treatment of the reactor with acetone made it possible to restore the productivity and extend the period of continuous operation for further 29 days.

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CHAPTER 4. Construction of Non-Support Bioreactor for Optical Resolution of 2-(4-Chlorophenoxy)-propanoic Acid in Organic Solvent Systems

INTRODUCTION

Enzymes are well known to express their catalytic activity even in organic solvents and various bioconversion of lipophilic or water-insoluble compounds in organic solvent systems have been carried out (1-3). However, there are only a few reports for bioreactors using organic solvents, in spite of the importance of continuously operated systems for production of useful substances in industrial fields (4-9). The author have demonstrated successful long-term continuous operation in the optical resolution of 2-(4-chlorophenoxy)propanoic acid by using water-saturated carbon tetrachloride-isooctane (8:2, v/v) in a column reactor packed with Celite-adsorbed lipase (an adsorbed bioreactor). The lipase maintained its activity in the organic solvent for more than 6 months (8).

In general, enzyme proteins are insoluble in organic solvents but can work even in a suspended state (9-11). This fact suggests that it will be possible to retain enzymes in reactors without immobilization, which is essential to keep biocatalysts in reactors with aqueous systems, to construct trailblazing "non-support bioreactor" with organic solvent systems.

The non-support bioreactor is thought to have many advantages; for example, simplicity for preparation, small volume of the reactor, and a low cost. Furthermore, it is expected to have high activity without inactivation of enzymes accompanied by immobilization and interference of diffusion to be caused by supports, resulting in a high productivity based on the reactor volume.

This chapter deals with the construction of a non-support bioreactor with an organic solvent system by selecting stereoselective esterification of (RS)-2-(4-chlorophenoxy)propanoic acid with 1-tetradecanol by lipase OF 360 from Candida cylindracea as a suitable model reaction (Fig. 1) (8, 12).

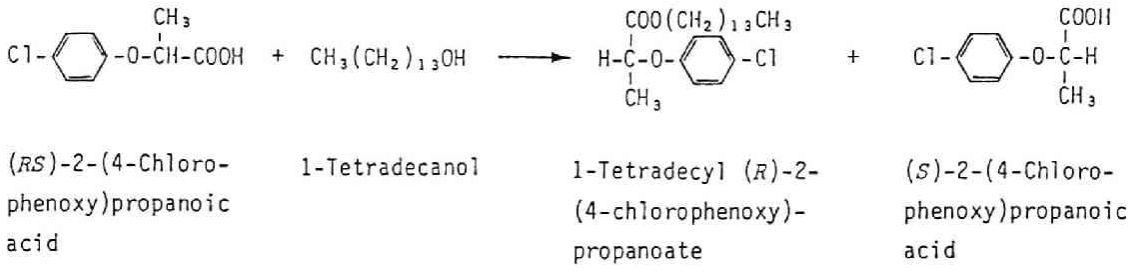

Fig. 1. Stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid with 1-tetradecanol by lipase OF 360.

MATERIALS AND METHODS

Materials

Lipase OF 360 from Candida cylindracea was purchased from Meito Sangyo, Tokyo, Japan, and used without further purification. (RS)-2-(4-Chlorophenoxy)propanoic acid was a product of Aldrich, Milwaukee, WI, USA, and 1-tetradecanol was obtained from Nacalai Tesque, Kyoto, Japan. All other chemicals were also obtained from commercial sources.

Batchwise reactions by non-supported and Celite-adsorbed lipase

Stereoselective esterification of 2-(4-chlorophenoxy)-propanoic acid with 1-tetradecanol by the yeast lipase was carried out at 30°C in a long-necked flask with shaking (120 strokes.min^{-1}). The reaction mixture was composed of 100 mg of free (non-supported) or Celite-adsorbed lipase OF 360 and 10 ml of water-saturated carbon tetrachloride-isooctane (8:2, v/v) containing 100 mM 2-(4-chlorophenoxy)propanoic acid and 100 mM 1-tetradecanol. Celite-adsorbed lipase was prepared in the same manner as described in CHAPTER 3.

Continuous and semi-continuous operation of non-support bioreactor

Continuous operation with non-supported lipase was carried out as follows: Free lipase OF 360 (10 g) was suspended in water-saturated carbon tetrachloride-isooctane (8:2, v/v) and the suspension was packed in a pressure-resistant glass column which had glass filters at the both ends (2 x 15 cm, Yamazen Co., Osaka, Japan). Then the same solvent containing each 100 mM of substrates

was soon supplied continuously from the bottom of the column at a flow rate of 173 ml.day^{-1} using a pump (Type L-6000; Hitachi, Tokyo, Japan) (Fig. 2A). Semi-continuous reaction was carried out by a column reactor which was packed 3 g of free lipase OF 360 by the same method mentioned above. After stabilizing the bed by flowing the substrate solution overnight at a flow rate of 173 ml.day^{-1}, 500 ml of the substrate solution was supplied from the bottom of the reactor and recycled at a high flow rate, 13 l.day^{-1} (Fig. 2B). In both cases, the suspended enzyme made stable beds and no outflow of the enzyme from the beds was occurred. Temperature of these reactors was maintained at 30^{o}C.

Analysis

The alcohol remained and the ester formed were determined by gas chromatography using a glass column (1.1 m) packed with silicon OV-17 supported on chromosorb W AW DMCS (carrier gas, N_2; flow rate, 60 ml.min^{-1}; injector temperature, 300^{o}C) by using n-icosane as an internal standard. With the use of temperature programs from 140^{o}C

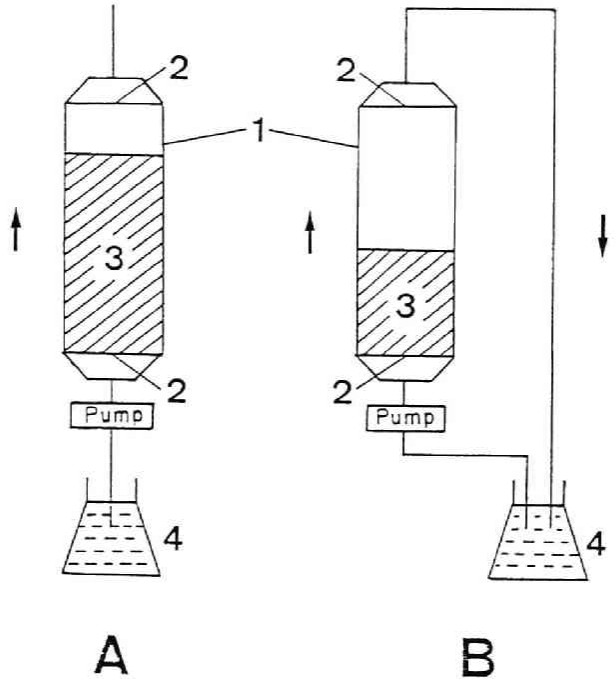

Fig. 2. Schematic diagrams of non-support bioreactors. A, Continuously operated system; B, semi-continuously operated system. 1, Pressure-resistant glass column (2 x 15 cm); 2, glass filter; 3, non-supported lipase OF 360; 4, substrate solution.

to 160°C at 4°C.min^{-1} and from 160°C to 280°C at 20°C.min^{-1}, the retention times observed were 4.5 min for 1-tetradecanol, 5.4 min for 2-(4-chlorophenoxy)propanoic acid, 7.2 min for n-icosane, and 11.6 min for 1-tetradecyl 2-(4-chlorophenoxy)propanoate. Optical purity of the remaining (S)-acid was determined from optical rotation, measured with a DIP-140 type polarimeter (Japan Spectroscopic Co., Tokyo, Japan), as described in CHAPTER 2.

RESULTS

Effect of support in batch reactions

Effect of support on the stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid with 1-tetradecanol by the yeast lipase in the organic solvent (water-saturated carbon tetrachloride-isooctane, 8:2, v/v) was investigated in batchwise reaction. As shown in Fig. 3, the non-supported lipase catalyzed the reaction three times faster than the Celite-adsorbed lipase at the initial phase of ester formation, probably due to the low

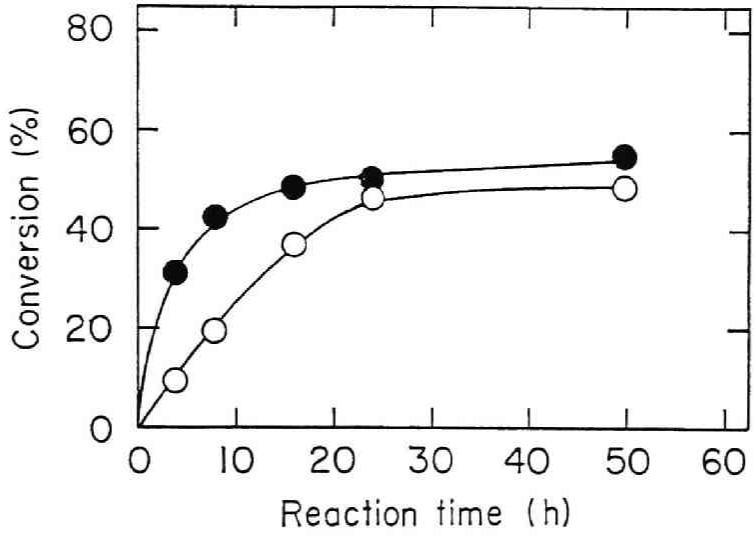

Fig. 3. Effect of support on stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid by lipase OF 360 in batchwise reaction. Water-saturated carbon tetrachloride-isooctane (8:2, v/v) was used as reaction solvent. (○), Lipase OF 360 (100 mg) adsorbed on 250 mg of Celite No. 535; (●), lipase OF 360 (100 mg) suspended directly in the solvent.

Table 1. Optical purity of the remaining acid in batchwise reaction

Lipase	Reaction time (h)	Conversion (%)	$[\alpha]_D^{25}$ [a] (deg)	% ee[b]
Celite-adsorbed	54	50.7	-30.0	75.8
Non-supported	20	51.4	-34.0	84.8

Stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid by Celite-adsorbed and non-supported lipase OF 360 was carried out with 1-tetradecanol in water-saturated carbon tetrachloride-isooctane (8:2, v/v).

[a] Specific rotation of the remaining acid.

[b] Enantiomeric excess of the remaining acid from the specific rotation with the value of $[\alpha]_D^{25}$ = -40.1 (c 1, ethanol) obtained from Beilstein.

interference of diffusion of the substrates and the products. Furthermore, optical purity of the remaining acid was similar in both cases (Table 1). These results suggested the possibility of constructing a highly active and highly stereoselective non-support bioreactor.

Continuous operation of non-support bioreactor

The author has tried continuous optical resolution of 2-(4-chlorophenoxy)propanoic acid in a non-support bioreactor, which is a column reactor packed with free lipase OF 360. When the lipase powder suspended in the organic solvent was packed in a glass column and the substrate solution was passed through from the top of the reactor, it was very difficult to get a steady flow because of the pressure drop caused by the tightly packed lipase powder. Therefore, the substrate solution was supplied from the bottom of the reactor by using pump immediately after introducing the lipase suspended in the organic solvent into the glass column (Fig. 2A).

It is essential to maintain the conversion ratio at 50 % for efficient optical resolution of a racemic compound.

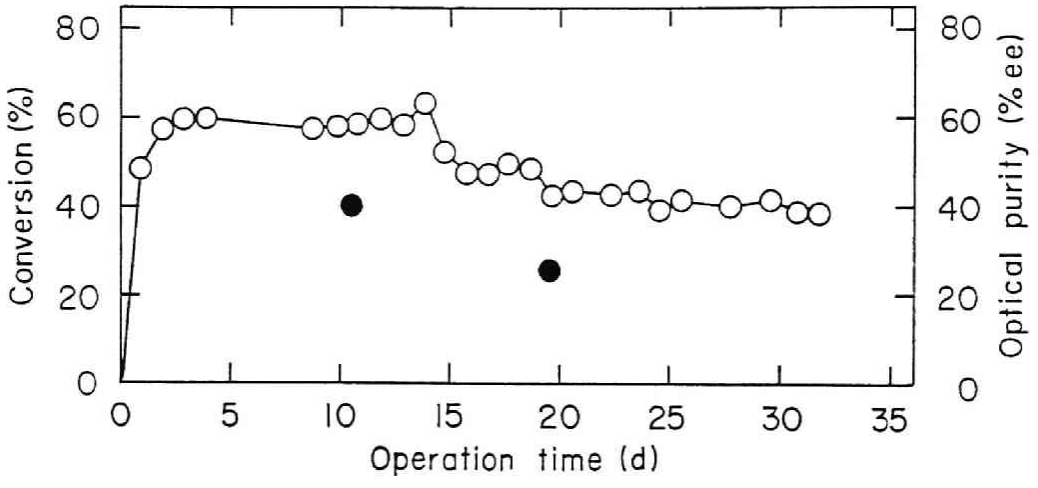

Fig. 4. Continuous stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid by a non-support bioreactor. Water-saturated carbon tetrachloride-isooctane (8:2, v/v) containing each 100 mM of the substrates was supplied from bottom of the column reactor packed with non-supported lipase OF 360 at a flow rate of 173 ml.day^{-1}. (○), Conversion (%); (●), enantiomeric excess of the remaining acid (% ee).

The continuous esterification of 2-(4-chlorophenoxy)-propanoic acid could be successfully carried out at approximately 50 % for 15 days by adjusting the flow rate of the substrate solution to 173 $ml.day^{-1}$ by the non-support bioreactor (Fig. 4). The conversion ratio did not reach 50 % at higher flow rates. The flow rate employed, 173 $ml.day^{-1}$, was twice of that in the adsorbed bioreactor, that is, a column reactor packed with the same amount of lipase after Celite-adsorption (CHAPTER 3). Furthermore, the bed volume of this non-support bioreactor was about a half because of "non-support". These results revealed that the non-support bioreactor exhibited four-fold productivity per system compared with the adsorbed bioreactor. However, the non-support bioreactor showed a low stereoselectivity toward the acid, being less than 40 % ee, even when the conversion ratio was kept at approximately 50 %. A lipase taken from middle part of the bed after continuous operation still retained a high stereoselectivity in batchwise reaction (Table 2).

Table 2. Stereoselectivity in batchwise reaction of non-supported lipase OF 360

Operation (Time)	Productivity[a] (μmol.h^{-1}.mg lipase $^{-1}$)	% ee[b] (Conversion)
1st batch (20 h)	0.17	84.8(51.4 %)
Continuous (763 h)	0.036	40.6(57.9 %)
2nd batch[c] (107 h)	0.056	86.8(53.7 %)

Stereoselective esterification of 2-(4-chlorophenoxy)-propanoic acid by non-supported lipase OF 360 was carried out with 1-tetradecanol in water-saturated carbon tetrachloride-isooctane (8:2, v/v).

[a] Ester formation rate.

[b] Enantiomeric excess of the remaining acid at indicated conversion ratio.

[c] The lipase recovered from the middle part of the bed after continuous operation for 763 h was used in batchwise reaction.

Semi-continuous production of optically active carboxylic acid by non-support bioreactor

As mentioned above, the non-support bioreactor did not show a high stereoselectivity in spite of the fact that the enzyme retained the specificity. This phenomenon might be explained by distortion of residence time distribution of the substrates caused by serious channeling and over-retention of the substrate solution through the packed enzyme at a low flow rate. Therefore, semi-continuous operation of the non-support bioreactor was tried by recycling the substrate solution at a high flow rate to eliminate this problem. Lipase (3 g) suspended in the organic solvent was packed in a glass column and the bed was stabilized by flowing the substrate solution overnight at a low rate. Then semi-continuous production of optically active 2-(4-chlorophenoxy)propanoic acid was started by supplying 500 ml of the substrate solution from the bottom of the reactor at a flow rate of 13 $l.day^{-1}$ with recycling. The bed volume of this reactor based on the lipase amount became two times as much as that

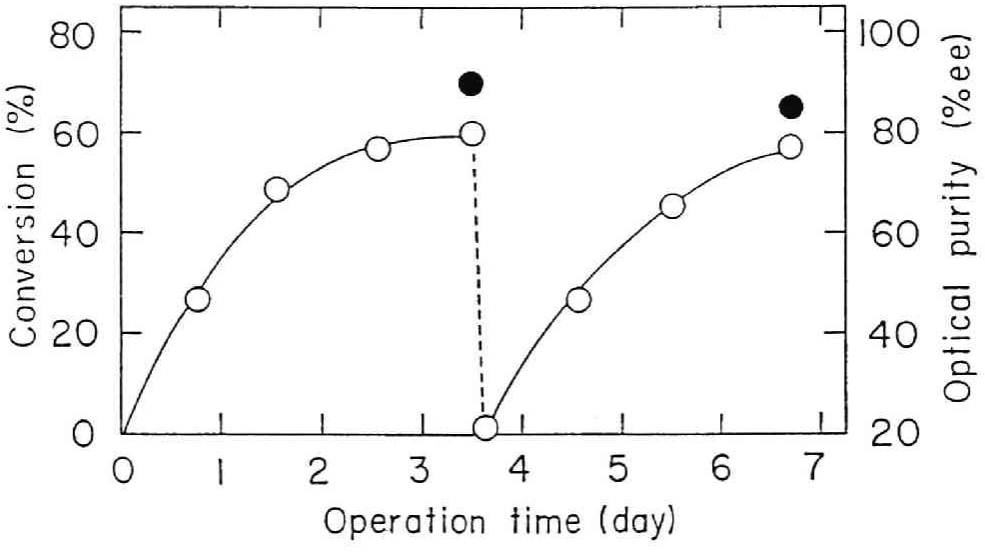

Fig. 5. Semi-continuous stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid by non-support bioreactor. Water-saturated carbon tetrachloride-isooctane (8:2, v/v) (500 ml) containing each 100 mM of the substrates was recycled by using a pump from bottom of the column reactor packed with non-supported lipase OF 360 with a flow rate of 13.0 $l.day^{-1}$. Broken line, the substrate solution was changed to the fresh one. (○), Conversion (%); (●), enantiomeric excess of the remaining acid (% ee).

of the continuously operated non-support bioreactor (Fig. 2B). The conversion ratio reached 50 % only after 2 days of reaction (Fig. 5) and the calculated productivity, that is, the ester formation rate, was 0.15 $\mu mol.h^{-1}.mg\ lipase^{-1}$. This is four-fold productivity per unit lipase compared with that of the continuously operated reactor (0.036 $\mu mol.h^{-1}.mg\ lipase^{-1}$). Furthermore, the optical purity of the remaining acid was high (85 % ee at 50 % conversion). Thus, the author could construct an efficient non-support bioreactor giving a high productivity and a high stereoselectivity.

DISCUSSION

Characteristics of the non-support bioreactor and the adsorbed bioreactor were summarized in Table 3. The non-support bioreactor operated continuously showed two-fold productivity per unit lipase due to its high activity without immobilization, and four-fold productivity per system because of its small volume of the bed without support. As for the stability, a half-life period of the

Table. 3 Comparison of productivity, stability, and stereoselectivity among various reaction systems

Reaction system	Productivity[a]		Stability		Stereoselectivity
	(μmol.h^{-1}. mg lipase^{-1})	(μmol.h^{-1}. ml bed^{-1})	Half-life (day)	Ester formed[b] (mol)	% ee[c] (Conversion)
Celite-adsorbed[d] (Continuous)	0.018	2.4	80	0.34	88.8(63.0%)
Celite-adsorbed[e] (Semi-continuous)	0.020	2.7	--	--	--
Non-supported[f] (Continuous)	0.036	9.5	39	0.35	40.6(57.9%)
Non-supported[g] (Semi-continuous)	0.15	23	--	--	89.5(59.6%)

(Footnote of Table 3)

Stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid by lipase OF 360 was carried out with 1-tetradecanol in water-saturated carbon tetrachloride-isooctane (8:2, v/v).

[a] Ester formation rate.

[b] Amount of the ester formed during half-life period.

[c] Enantiomeric excess of the remaining acid at indicated conversion ratio.

[d] 10 g of lipase (Celite-adsorbed) and down-flow of 86.4 $ml.day^{-1}$ (bed volume, 75 ml). Other conditions were described in CHAPTER 3.

[e] 10 g of lipase (Celite-adsorbed) and up-flow of 8.64 $l.day^{-1}$ (bed volume, 75 ml). Other conditions were described in CHAPTER 3.

[f] 10 g of lipase and up-flow of 173 $ml.day^{-1}$ (bed volume, 38 ml).

[g] 3 g of lipase and up-flow of 13.0 $l.day^{-1}$ (bed volume, 20 ml).

non-support reactor (39 days) with respect to the conversion ratio was a half of that of the adsorbed bioreactor, while the amounts of the ester formed during the half-life period in both cases were almost equal. The author can say that the lipase was stable in the organic solvent even without immobilization. Although the continuously operated non-support bioreactor did not show a high stereoselectivity, it was enough effective on fast bioconversion. However, it was possible to remove this disadvantage by semi-continuous operation and obtain further two-fold productivity with a high stereoselectivity.

In this study, the author successfully constructed a "non-support bioreactor" which has never been reported and revealed that the non-support bioreactor had many advantages, such as simplicity for preparation, small volume, a low cost, and a high activity and productivity. Such a reactor may be applicable to the production of other useful substances by enzymatic reaction using organic solvent systems.

SUMMARY

"Non-support bioreactor", a novel column reactor packed with a free non-supported enzyme was constructed by applying insolubility of the enzyme in organic solvents. Stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid by lipase OF 360 from Candida cylindracea with 1-tetradecanol was selected as a model reaction. Non-supported lipase revealed three-fold higher activity than Celite-adsorbed lipase by keeping a high stereoselectivity in a batchwise reaction. In continuous operation, a non-support bioreactor produced the ester with four-fold productivity to that of a column reactor packed with Celite-adsorbed lipase (an adsorbed bioreactor). However, optical purity of the remaining (S)-acid was low even when the conversion ratio was kept at approximately 50 %. Lipase recovered from the non-support bioreactor after continuous operation retained the original stereoselectivity in a batchwise reaction. Therefore, semi-continuous operation was conducted by recycling the substrate solution at a high flow rate. The non-support reactor showed a high stereose-

lectivity and 10 times the productivity compared with the adsorbed bioreactor. The reason of this high performance was also discussed.

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CHAPTER 5. Efficient Optical Resolution of 2-(4-Chlorophenoxy)propanoic Acid by the Use of Organo-Silicon Compounds as Substrates of Lipase

INTRODUCTION

Increasing attention is being focused on the potential of enzymes in synthetic organic chemistry (1-2). The utility of various kinds of hydrolases has been reported for efficient esterification in organic solvent media (3) and it was found that lipase OF 360 from Candida cylindracea esterified several terpene alcohols stereoselectively (4). The author has also successfully carried out the stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid by lipase OF 360 in an organic solvent and revealed that chain-length of alcohols as the acyl acceptors markedly affected both the reaction rate and the stereoselectivity (5). However, many problems remain to be solved in the enzymatic processes. For example, it is often quite difficult to construct a highly stereoselective bioconversion system with a high reaction rate, which is a

very important prerequisite for industrial application of enzymes, by the use of conventional compounds as substrates.

To break through these problems and raise the potential of enzymes, use of unconventional compounds, such as non-natural organosilicon compounds, will be attractive. Furthermore, fundamental studies on the modes of recognition and catalysis of enzymes toward such the non-natural compounds seem to be of great interest and importance.

Organosilicon compounds have specific characters from the viewpoints of comprehensive organic chemistry (6), and the production of organosilicon compounds, useful as bioactive compounds or their precursors, by effective catalysts such as enzymes is of great importance.

Therefore, a combination of organosilicon chemistry with biochemistry will be very attractive. However, only a few papers are available for the bioconversion of organosilicon compounds (7-9). Hitherto, there have been no study on the ability of organosilicon compounds to break the limit of conventional substrates and on the modes of recognition and reaction of enzymes for organosilicon compounds as compared with conventional substrates.

In this work, the author has attempted to use the organosilicon compounds ($Me_3Si(CH_2)_nOH$) having different chain-length between trimethylsilyl group and hydroxyl group in the esterification process to construct an efficient optical resolution system which is difficult with conventional substrates (Fig. 1). The enzymatic recognition of organosilicon compounds, comparing with the corresponding carbon compounds ($Me_3C(CH_2)_nOH$), was also discussed.

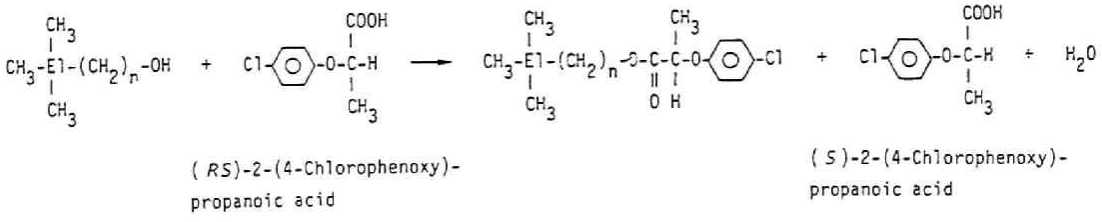

Fig. 1. Stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid with organosilicon compounds and the corresponding carbon compounds. El = Si or C

MATERIALS AND METHODS

Enzymes

The following three kinds of lipases were used: Lipase OF 360 (*Candida cylindracea*, Meito Sangyo Co., Tokyo, Japan), Lipase Saiken 100 (*Rhizopus japonicus*, Osaka Saikin Kenkyusho, Osaka, Japan), Lipase (Steapsin) (hog pancreas, Tokyo Kasei Kogyo Co., Tokyo, Japan).

Chemicals

Trimethylsilanol and trimethylsilylmethanol were obtained from Shin-Etsu Kagaku Kogyo Co., Tokyo, Japan. 2-Trimethylsilylethanol, 3-trimethylsilylpropanol, and 3,3-dimethylbutanol were purchased from Aldrich Chemical Co., Milwaukee, WI, USA. 1,1-Dimethylethanol and Celite NO. 535 were purchased from Wako Pure Chemical Industries, Osaka, Japan. 2,2-Dimethylpropanol was purchased from Nacalai Tesque, Kyoto, Japan. Other chemicals were also obtained from commercial sources.

Adsorption of lipases on Celite

Lipase (100 mg) suspended in 100 µl of deionized water was mixed thoroughly with 250 mg of Celite No. 535.

Enzymatic reactions

The reactions were carried out at 30 ^{o}C with shaking (120 strokes.min^{-1}). The reaction mixtures were composed of Celite-adsorbed lipase (corresponding to 100 mg of lipase) and 10 ml of water-saturated benzene containing 100 mM (RS)-2-(4-chlorophenoxy)propanoic acid and 100 mM alcohol.

Analysis

An aliquot (50 µl) of the reaction mixtures was added to 50 µl of ethanol containing a known amount of n-pentadecane as an internal standard. The amount of esters produced was measured by gas chromatography using a glass column packed with PEG-HT supported on Uniport R (carrier gas, N_2; detector, FID). 2-(4-Chlorophenoxy)propanoic acid remaining after the reactions was isolated from the reaction mixtures by column chromatography (Silica gel 60, 70-230 mesh). Optical purity was determined from optical rotation measured with a DIP-140 type polarimeter (Japan Spectroscop-

ic Co.,Tokyo, Japan).

RESULTS AND DISCUSSION

Four organosilicon compounds, $Me_3Si(CH_2)_nOH$, which have different chain-length between trimethylsilyl group and hydroxyl group (n = 0, 1, 2, and 3), and three corresponding carbon compounds, $Me_3C(CH_2)_nOH$ (n = 0, 1, and 2), were examined as acyl acceptors in the stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid using lipase OF 360 as biocatalyst in water-saturated benzene (Fig. 1).

Organosilicon compounds and the carbon counterparts examined in this study, except for the case of n = 0 (trimethylsilanol and 1,1-dimethylethanol), served as acyl acceptors with high to moderate stereoselectivity in esterification of (R)-2-(4-chlorophenoxy)propanoic acid with lipase OF 360 (Table 1). When these organosilicon compounds were used as substrates, enzyme activity increased in the following order; n = 0, n = 3, n = 2, n = 1. Trimethylsilylmethanol (n = 1) was found to be particularly an excellent

Table 1. Stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid with organosilicon compounds and the corresponding carbon compounds by lipase OF 360

Alcohol	n	Reaction time (h)	Conversion (%)	% ee[a]
Trimethylsilanol	0	168	nil	----
1,1-Dimethylethanol	0	168	nil	----
Trimethylsilylmethanol	1	19	51.7	95.8
2,2-Dimethylpropanol	1	196	52.8	91.1
2-Trimethylsilylethanol	2	24	52.2	76.1
3,3-Dimethylbutanol	2	21	51.8	71.6
3-Trimethylsilylpropanol	3	44	51.0	77.5

[a] % ee of remaining 2-(4-chlorophenoxy)propanoic acid

substrate for the esterification -- that is, the reaction rate was extremely higher than that with 2,2-dimethylpropanol (n = 1) and the enantiomeric excess of the acid remained was above 95 % ee. These results are quite superior to those with 1-tetradecanol as an acyl acceptor (reaction time at about 50 % conversion, 72 h; 82 % ee) (CHAPTER 2). It is interesting that, in the cases of conventional substrates, a high reaction rate did not consist with a high stereoselectivity as observed on the esterification of 2-(4-chlorophenoxy)propanoic acid with branched-chain alcohols (Table 1) and linear-chain alcohols (CHAPTER 2) and on the esterification of menthol with carboxylic acids (10). The results obtained in this study indicate that non-natural substrates may solve the unavoidable and probably inherent problems on stereoselective reactions with conventional substrates. In fact, application of organosilicon compounds as non-natural substrates enabled such rapid and highly stereoselective esterification as described above, which has never been attained with the conventional alcohols.

Fig. 2 shows the time-course of esterification of 2-(4-chlorophenoxy)propanoic acid with the organosilicon and the

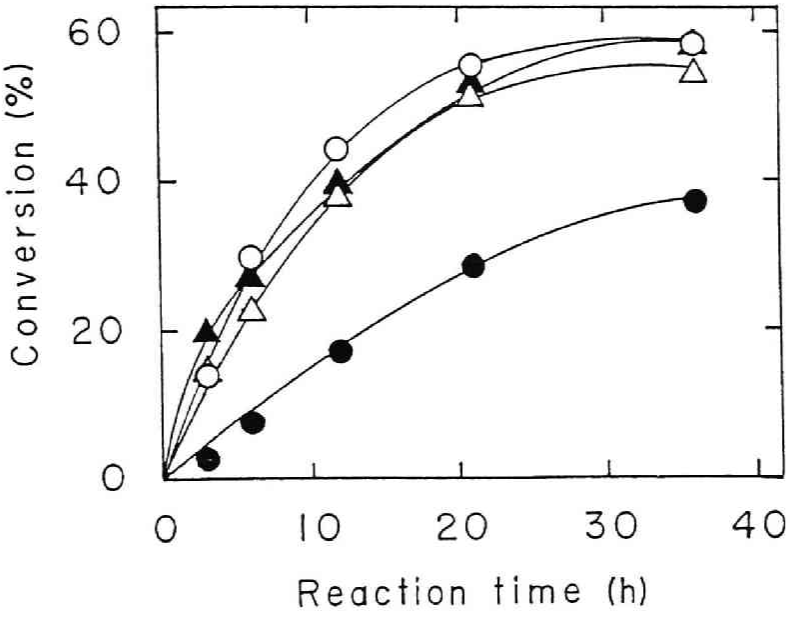

Fig. 2. Time-course of esterification of 2-(4-chlorophenoxy)propanoic acid with organosilicon compounds and the corresponding carbon compounds. (○), trimethylsilylmethanol (n = 1); (●), 2,2-dimethylpropanol (n = 1); (△), 2-trimethylsilylethanol (n = 2); (▲), 3,3-dimethylbutanol (n = 2)

corresponding carbon compounds (n = 1, 2) by lipase OF 360 in water-saturated benzene. It was found that trimethylsilylmethanol (n = 1) was esterified faster than the carbon analogue (n = 1) and the reaction stopped at 50 - 60 % conversion. These results have shown that the trimethylsilylmethanol (n = 1) reacted fast with (R)-2-(4-chlorophenoxy)propanoic acid stereoselectively. In the case of n = 2, there was no difference in the conversion profiles between the organosilicon and the corresponding carbon compounds (Table 1 and Fig. 2). Thus, silicon atom was regarded as a mimic carbon atom by lipase OF 360 in the case of 2-trimethylsilylethanol (n = 2), but made trimethylsilylmethanol (n = 1) an excellent substrate for stereoselective esterification with lipase.

Physical properties of silicon atom and carbon atom are shown in Table 2 (6). Electronegativity of silicon atom is lower than that of carbon atom. This fact might result in higher nucleophilicity of oxygen atom of trimethylsilylmethanol (n = 1) compared with that of 2,2-dimethylpropanol (n = 1), and the hydroxyl group of trimethylsilylmethanol is less sterically hindered due to bigger

Table 2. Physical properties of silicon and carbon atoms

Atom	Electronegativity	Bond	Bond length (nm)
C	2.55	C-C	0.153
Si	1.90	Si-C	0.189

atomic radius of silicon atom than that of carbon atom in 2,2-dimethylpropanol, as illustrated in Fig. 3. Trimethylsilylmethanol might, therefore, be more easily accessible to the acyl-enzyme intermediate, and react as an acyl acceptor much faster than 2,2-dimethylpropanol. In fact, K_m value of lipase OF 360 for trimethylsilylmethanol was smaller than that for the counterpart and V_{max} value was increased (Table 3). Probably the effect of silicon atom mentioned above are not favorable in 2-trimethylsilylethanol (n = 2) owing to

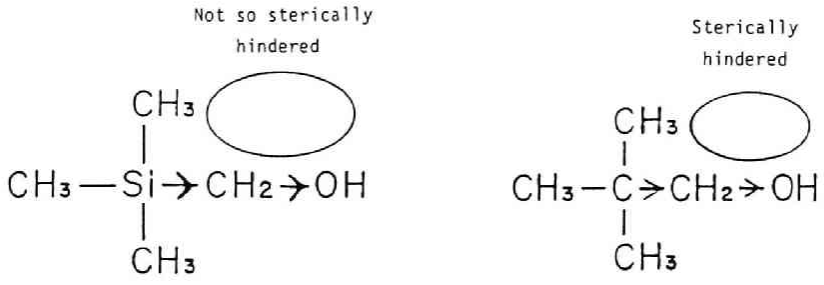

Fig. 3. Effects of silicon atom in trimethylsilylmethanol (n = 1).

Table 3. Kinetic parameters for esterification with trimethylsilylmethanol and 2,2-dimethylpropanol by lipase OF 360

Alcohol	K_m (mM)	V_{max} ($\mu mol.h^{-1}.mg\ powder^{-1}$)
Trimethylsilylmethanol	132	1.7
2,2-Dimethylpropanol	227	0.8

Kinetic parameters were measured with 100 mM 2-(4-chlorophenoxy)-propanoic acid.

the presence of long ethylene group between silicon atom and hydroxyl group. 2-Trimethylsilylethanol might be consequently regarded as similar substrate to 3,3-dimethylbutanol (n = 2) by lipase. In spite of the favorable behavior of trimethylsilylmethanol for the esterification, silicon provided no specific effect on the esterification of trimethylsilanol (n = 0) (Table 1). Several lipases from different sources were also examined concerning the interesting phenomena observed here. All lipases tested could esterify the acid faster with trimetylsilylmethanol than with the corresponding carbon compound (Table 4). These results indicate that the specific character of silicon atom in trimethylsilylmethanol (n = 1) would play important roles in the molecular recognition of the lipase family.

In this work, the author showed that the use of organosilicon compounds as non-natural substrates for enzymes not only improved the ability of the enzymes but also gave a clue to study the modes of recognition and reaction of enzymes such as lipases. Application of trimethylsilylmethanol as a substrate enabled the fast and

highly stereoselective reaction which was difficult with conventional substrates. Furthermore, it was found that silicon atom served not only as a mimic carbon atom in 2-trimethylsilylethanol (n = 2) but also as an effective atom to enhance the reactivity of trimethylsilylmethanol (n = 1) in the enzymatic recognition.

Table 4. Esterification of 2-(4-chlorophenoxy)propanoic acid with trimethylsilylmethanol and 2,2-dimethylpropanol by lipases from different sources

Lipase	Source	Reaction rate ($\mu mol \cdot h^{-1} \cdot mg\ powder^{-1}$)	
		Trimethylsilylmethanol	2,2-Dimethylpropanol
Lipase OF 360	Candida cylindracea	0.40	0.15
Lipase Saiken 100	Rhizopus japonicus	0.12	0.02
Lipase (Steapsin)	Hog pancreas	0.03	0.01

SUMMARY

Comparative studies were made on the utility as acyl acceptors of organosilicon compounds ($Me_3Si(CH_2)_nOH$) and the corresponding carbon compounds ($Me_3C(CH_2)_nOH$) in the stereoselective esterification of 2-(4-chlorophenoxy)-propanoic acid by the use of lipase OF 360 of Candida cylindracea in water-saturated benzene. The organosilicon compounds were effectively used as the substrates for the esterification of the (R)-acid enantiomer. Of the organosilicon compounds of different chain-length between the silicon atom and the hydroxyl group, trimethylsilylmethanol (n = 1) enabled the esterification reactions both fast and highly stereoselective, which were difficult with conventional substrates such as its carbon counterpart. On the other hand, no difference was observed between 2-trimethylsilylethanol (n = 2) and its carbon analogue (3,3-dimethylbutanol) in the enzymatic activity and the stereoselectivity. These results indicate that the silicon atom behaved as a mimic carbon atom for lipase in the case of 2-trimethylsilylethanol but was effective to

enhance the reactivity of trimethylsilylmethanol. The differences could be explained on the basis of the properties of silicon atom, such as low electronegativity and big atomic radius compared with carbon atom.

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GENERAL CONCLUSION

The present studies dealt with the stereoselective esterification of carboxylic acids by hydrolases in organic solvent systems to construct an efficient bioconversion system for the optical resolution of carboxylic acids, especially 2-(4-chlorophenoxy)propanoic acid whose (R)-enantiomer is an important herbicide. Continuous or semi-continuous optical resolution of 2-(4-chlorophenoxy)propanoic acid was successfully carried out not only by a column reactor packed with Celite-adsorbed lipase OF 360 but also by a non-support bioreactor, a novel column reactor packed with free non-supported lipase OF 360, in organic solvent system. Furthermore, the author revealed that organosilicon compounds were good unconventional substrates for efficient optical resolution of 2-(4-chlorophenoxy)propanoic acid and showed that unconventional substrates such as organosilicon compounds might solve the unavoidable and probably inherent problems of stereoselective reactions with conventional substrates.

First, fifty different Celite-adsorbed hydrolases were

screened for their esterification activity in an organic solvent with citronellol and 5-phenylpentanoic acid as substrates. Twenty two hydrolases were found to be very active as catalysts in the organic solvent. Furthermore, the effects of reaction conditions on esterification activity were studied by using lipase OF 360 from Candida cylindracea, which was selected based on the availability and activity. Water content in supports used for the enzyme immobilization had a significant effect on the enzymatic activity. There was the optimum water content for activity of lipase OF 360. Hydrophobic organic solvents such as isooctane and cyclohexane were found to be suitable for esterification by lipase OF 360, whereas polar organic solvents such as acetone and chloroform caused reversible inactivation of the enzyme.

Second, optical resolution of halogen-containing carboxylic acids was attempted with batchwise stereoselective esterification by Celite-adsorbed hydrolases. Lipase OF 360 was found to stereoselectively esterify 2-(4-chlorophenoxy)propanoic acid in water-saturated benzene, and the effect of the chain length

of alcohols as second substrates on activity and stereoselectivity of lipase OF 360 were investigated. The results revealed that the alcohol chain length remarkably affected the stereoselective esterification of 2-(4-chlorophenoxy)-propanoic acid. Alcohols of shorter chain length served as excellent acyl acceptors for esterification, whereas longer chain alcohols were not so good. On the contrary, sufficient stereoselectivity towards 2-(4-chlorophenoxy)propanoic acid was not observed with shorter chain alcohols in contrast to the high stereoselectivity with longer chain alcohols. For further studies, the author selected 1-tetradecanol as an acyl acceptor because stereoselectivity was high and reaction rate was moderate.

Long-term continuous optical resolution of 2-(4-chlorophenoxy)propanoic acid was achieved by stereoselective esterification with 1-tetradecanol using a column reactor packed with Celite-adsorbed lipase OF 360 in an organic solvent system. Water-saturated carbon tetrachloride-isooctane (8:2, v/v) was an excellent organic solvent for continuous operation, but the particle size of Celite did not affect the productivity. Under the optimized conditions, (R)-enantiomer of the acid was continuously esterified with

high stereoselectivity by lipase OF 360 packed in the column reactor for 34 days. Furthermore, treatment of the reactor with acetone to remove water formed made it possible to restore the activity and extend the period of continuous operation for further 29 days.

To construct more efficient bioreactor, the author designed a non-support bioreactor, which is a novel column reactor packed with a free non-supported enzyme by applying the insolubility of the enzymes in organic solvents. Continuous stereoselective esterification of 2-(4-chlorophenoxy)propanoic acid with 1-tetradecanol was attempted by the use of the non-support bioreactor packed with free lipase OF 360 in an organic solvent system. The non-support bioreactor showed four-fold higher productivity than that of the column reactor packed with Celite-adsorbed lipase OF 360 (the adsorbed bioreactor) in continuous operation, but the optical purity of the remaining (S)-acid was reduced to less than 40 % ee even when the conversion ratio was kept at nearly 50 %. However, the non-support bioreactor exhibited not only ten times the productivity compared with the adsorbed bioreactor but also high

stereoselectivity in the case of semi-continuous operation. The non-support bioreactor had many advantages such as simplicity of application of enzymes without immobilization, small volume of the reactor, high productivity, and low cost. Therefore, the non-support bioreactor will be in the limelight as one of the most promising bioreactors.

It was found that trimethylsilylmethanol enabled the esterification of 2-(4-chlorophenoxy)propanoic acid to be both fast and highly stereoselective which was difficult with conventional substrates such as their carbon counterparts and 1-tetradecanol. On the contrary, no difference was observed between 2-trimethylsilylethanol and its carbon analogue in reaction rate and stereoselectivity. These results indicated that the silicon atom behaved as a mimic carbon atom for lipase in the case of 2-trimethylsilylethanol but was effective in enhancing the reactivity of trimethylsilylmethanol. From these results, it was concluded that the use of unconventional substrates such as organosilicon compounds would extend the possibility of enzymes and might solve the unavoidable problems of stereoselective esterification with conventional substrates.

The author believes that the study summarized here will

develop new ability of enzymes and will bring further expansion of application of enzymes.

PUBLICATION LIST

CHAPTER 1.

1) Esterification in organic solvents: selection of hydrolases and effects of reaction conditions.
Biocatalysis, 1, 137-145 (1987)

CHAPTER 2.

2) Stereoselective esterification of halogen-containing carboxylic acids by lipase in organic solvent: effects of alcohol chain length.
Appl. Microbiol. Biotechnol., 34, 47-51 (1990)

CHAPTER 3.

3) Long-term continuous production of optically active 2-(4-chlorophenoxy)propanoic acid by yeast lipase in an organic solvent system.
Appl. Microbiol. Biotechnol., 34, 330-334 (1990)

CHAPTER 4.

4) Construction of non-support bioreactor: optical resolution of 2-(4-chlorophenoxy)propanoic acid in an organic solvent system.
Appl. Microbiol. Biotechnol., 35, 563-567 (1991)

CHAPTER 5.

5) Organo-silicon biochemistry.
Ann. N. Y. Acad. Sci., 613, 702-706 (1990)

6) Efficient optical resolution of 2-(4-chlorophenoxy)-propanoic acid with lipase by the use of organosilicon compounds as substrate: the role of silicon atom in enzymatic recognition.
J. Biotechnol., 18, 85-92 (1991)

BOOK

1) Immobilized enzymes in organic solvents.
In Protein Immobilization, R. F. Taylor (ed.), pp.183-208, Marcel Dekker, Inc., New York, NY, USA (1991)